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Immunoassay of alkaloids

S K Volkov

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Abstract. A survey is presented of literature data concerning the development and application of the immunochemical methods for the assay of alkaloids — one of the classes of physiologically active compounds of plant origin most widely used in medical practice. The bibliography includes 141 references.

I. Introduction

Alkaloids are nitrogen-containing organic compounds which are encountered in plants and which as a rule exhibit physiological activity. Similar compounds have been detected also in certain animal organisms, in higher and lower fish, and in algae. More than 5000 different alkaloids have now been described and the chemical features of many of them have been fully established.

One of the most important branches of the modern science of medicinal plants involves the analytical determination of the content of biologically active substances in plant tissues. Not only the economic parameters of the manufacture of a number of medicinal preparations but also their quality depend on the content of physiologically active substances in the plant raw material.

Another important application of the analytical methods is the study of the biosynthesis of alkaloids in plant tissues.

Furthermore, analytical methods are widely used in the study of the metabolism and pharmacokinetics of the alkaloids used in medical practice.

A series of alkaloids possess narcotic properties, so that the rapid and reliable detection of these compounds in the human organism is very important from the medical point of view. Assay of narcotics is important also from the criminological standpoint.

The methods of synthesis of biologically active compounds in cell and plant tissue cultures have been vigorously developing in recent years. Theoretically this may make it possible to abandon the laborious operations involving the collection of plant material, especially wild species, and the use of large areas of fertile soil for the cultivation of such plants. The successful development of biotechnological methods is also associated with the analytical determination of the content of the target substances.

S K Volkov, The 'All-Russian Institute of Medicinal and Aromatic Plants' Scientific-Industrial Association, ul. Grina 7, 113628 Moscow.
Tel. (7-095) 388 45 22

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The necessity for a large number of analyses, especially in selection studies, has given rise to the need for the automation of analytical methods.

The methods for the quantitative assay of the substances present in plants can be divided into three groups: photometric, chromatographic, and protein-binding.

The photometric analytical methods have found the greatest application in the assay of biologically active substances of plant origin. These methods are relatively insensitive and non-specific, but, by virtue of their simplicity, they are still being used.

Chromatographic methods [thin-layer chromatography (TLC), gas–liquid chromatography (GLC), chromatography-linked mass spectrometry (GLC-MS), and high performance liquid chromatography (HPLC)] make it possible to determine fairly rapidly and accurately several substances being assayed in a single sample. However, these methods are fairly laborious. The most accurate and reliable of these is GLC-MS, but the application of this method is restricted by the high cost and complexity of the assay, which requires specially trained service personnel.

The protein-binding methods include the receptor and immune methods. The receptor assay method is based on the competitive inhibition by the compound assayed of the binding of a radioactively labelled ligand by membrane receptors specific to it.[1–3] This method is hardly used in the assay of substances present in plants. The reason for this is the difficulty in the identification and isolation of specific receptors.

The immunoassay methods are based on the specific interaction of the molecules of the substance assayed with the antibodies produced by the animal organism in response to the entry of foreign substances into the latter.

The protein-binding and in particular immune methods have a number of advantages over the photometric and chromatographic analytical methods: they do not require a large amount of work on the preparation of the specimen to be analysed, are highly sensitive, do not need complex and expensive equipment, and permit the simultaneous analysis of a large number of specimens.

The principal disadvantage of the immunoassay methods for low-molecular-mass substances is the necessity for preliminary work associated with the synthesis and purification of the antigen, the immunisation and maintenance of the animals, and the isolation, purification, and characterisation of the antibodies. Another significant disadvantage of the immunoassay methods is the possibility of the cross-interaction of the antibodies with a number of compounds having a structure similar to those of the antigen.

II. Immune methods for the assay of low-molecular-mass compounds

A fairly large number of reviews and monographs have been devoted to the immunoassay methods. They deal in detail with various procedures whereby the analyses are set up and carried out.[4-14]

There exist at present fairly numerous versions of immunoassay. The heterogeneous immunoassay methods used in the assay of low-molecular-mass compounds are as follows: radioimmunoassay, direct competitive enzyme immunoassay, and indirect enzyme immunoassay using secondary antibodies.

In radioimmunoassay (RIA), a standard amount of a radioactively labelled hapten is mixed with the specimen analysed. The radioactive hapten and the molecules of the specimen interact competitively with the antibodies, the degree of binding of the radioactive hapten to the antibodies being inversely proportional to the number of molecules assayed in the specimen. After equilibration, the hapten molecules bound to the antibodies are separated from the remaining free molecules either by the rapid adsorption of the haptens or by the precipitation of the proteins, after which the radioactivity of a particular phase is measured.

In the direct competitive enzyme immunoassay (EIA), an enzyme label and not a radioactive label is used. Antibodies present in a limited amount are bound to the solid phase, usually a polystyrene test tube or the walls of the microtitration tray. Competition between the substance assayed in the specimen and the enzyme-bound hapten leads to the immobilisation of a proportion of the enzyme on the solid phase. The free enzyme is removed and the amount of bound enzyme is determined, after adding the substrate, from the intensity of the colour of the solution caused by the substrate decomposition product.

In the analysis by the indirect immunoassay method using secondary antibodies [enzyme-linked immunosorbent assay (ELISA)], the hapten conjugated with the protein is sorbed on a solid phase — usually on the walls of the microtitration tray. The conjugate with which the animal was immunised is not used in this stage; instead one employs a conjugate with a protein unrelated to that of the immunogenic conjugate. Next the substance to be assayed, containing the free hapten and the antiserum, is added. The antibodies are distributed between the free hapten of the specimen and the immobilised hapten–protein conjugate. The liquid is decanted and an excess of secondary antibodies conjugated to the enzyme and specific to the immunoglobulins G (Ig G) of the immunised animal is added. The secondary antibodies are bound to the primary antibodies and are retained on the solid phase. The liquid phase is again decanted and the amount of secondary antibodies is determined after adding the substrate to the conjugated enzyme and the formation of the coloured product.

The homogeneous immunoassay methods most frequently used are those based on the measurement of the degree of polarisation of the fluorescence and the change in the activity of the enzyme.

In the immunoassay based on the measurement of the degree of polarisation of the fluorescence [fluorescence-polarisation immunoassay (FPIA)], a hapten conjugated to fluorescein or another molecule which fluoresces when irradiated with a beam of polarised light is employed. In the free state, the hapten–fluorescein conjugate in solution is mobile and the degree of polarisation of the light transmitted through the solution is low. In the form bound to the antibodies, the mobility of the hapten–fluorescein conjugate is restricted, which increases the degree of polarisation of the light. The amount of the hapten in the specimen is determined from the degree of substitution by the latter of the hapten–fluorescein conjugate in the interaction with the antibodies, which lowers the degree of polarisation of the light.

In the immunoassay based on the change in the activity of the enzyme [enzyme-multiplied immunoassay (EMIA)], one uses a hapten conjugated with the enzyme in such a way that the binding of the hapten to the antibodies lowers the activity of the enzyme owing to the blocking of its active centre. Thus the degree of recovery of the enzyme activity of the specimen analysed in the presence of the free hapten permits the determination of its amount.

Each of these methods has its advantages and disadvantages. Radioimmunoassay requires the synthesis of the radioactively labelled hapten. Fairly expensive equipment is also necessary — a centrifuge which permits the simultaneous centrifugation of up to 100–200 specimens and a liquid-scintillation (in the case of the ^{3}H isotope) or a γ-emission (in the case of the ^{125}I isotope) counter. The advantage of the ^{3}H isotope consists in the fact that the native structure of the molecule is retained.

The fluorescent and chemiluminescent labels, which make it possible to avoid the use of radioactive isotopes, appreciably alter the native structure of the hapten. These methods also require expensive equipment.

The use of enzyme labels in the stationary phase in the direct competitive enzyme immunoassay simplifies the analysis, since the phases can be separated very simply and only a colorimeter is required for the quantitative determination. However, this method requires the synthesis of the hapten–enzyme conjugate and the selection of the necessary hapten/enzyme ratio. Furthermore, owing to the incomplete adsorption of the enzyme, the antiserum — the most important component in the method — is not consumed in a rational manner. Another disadvantage of this method is that the enzyme and the specimen analysed, which frequently contains a multiplicity of components, are incubated together, which can lead to the inactivation of the enzyme. Furthermore the homogeneous methods are also less sensitive.

Direct immunoassay with the use of secondary antibodies is free from many of the above disadvantages. In this method, the antiserum is consumed fairly economically, since only dilute solutions are used. The high affinity and specificity of the antibodies make it possible to achieve a high sensitivity and specificity of the assay. Although in this method there is an additional stage involving incubation with the secondary antibodies, the employment of the latter makes it possible to increase the sensitivity of the assay and to extend the range of the measurable concentrations. The conjugates of enzymes with secondary antibodies to immunoglobulins from various sources are fairly readily commercially available. This method is the most popular and perhaps the best of all the immune methods which exist at the present time. The highest sensitivity of the method, attained in the analysis of the cholera toxin, amounts to 10^{-16} g, i.e. 600 molecules of this toxin is sufficient for the determination.[15]

The principal advantage of the homogeneous immunoassay methods is that, by performing the assay in one phase, it is possible to automate it and this makes them particularly attractive for routine assays of large numbers of specimens. It is more difficult to automate heterogeneous analytical methods, although here too the use of laboratory robots makes it possible to attain a productivity of up to 1000 specimens per day.

Analysis of the studies published during the period from 1959 to 1982 revealed a change in 1979–1981 from the radioimmunoassay to the enzyme immunoassay methods, which have virtually predominated since 1985.[14]

Analysis of chemical compounds by immune methods presupposes the employment of antibodies which recognise specifically the compounds to be analysed. However, substances with a molecular mass below 1000, including alkaloids, do not induce an immune response and formation of antibodies when they enter an animal organism. In order to stimulate an immune response, such substances must be bound covalently to large molecules (usually to a protein); the resulting conjugates are then used to immunise the animals. The antiserum produced after immunisation contains a mixture of antibodies, some of which may prove to be capable of recognising the hapten not only in the bound state but also in a free state.

A number of methods based on the use of various functional groups of the hapten have been proposed for the synthesis of immunogenic conjugates. The specificity of the antibodies thus obtained is determined by the sections of the molecule most remote from the conjugation point.[16] Careful study of the structure and properties of the hapten molecule and the correct selection of the conjugation point make it possible in many instances to obtain an antiserum with the required selectivity.[17] Many natural products contain several functional groups, which permits in many instances the variation of the way in which the conjugation is achieved. If there is no suitable group, it is possible to employ derivatives containing a functional group in the required site in the molecule.

Carboxy- or hydroxy-groups of the hapten frequently participate in the formation of the immunogenic conjugate. When the protein molecule is bound to the hapten via the carboxy-group, a two-stage synthesis takes place as a rule. In the first stage, a derivative of the hapten with a free carboxy-group is obtained with the aid of a bifunctional reagent. The mixed anhydride and carbodiimide methods are used for this purpose.

The mixed anhydride method,[18] widely known in organic chemistry, has been used to obtain ajmalicine conjugates as early as 1959,[19] while the carbodiimide method,[23] proposed for the synthesis of immunogenic conjugates in 1981, has been used to obtain the conjugates of serpentine,[19] cotinine,[20] vinblastine, vincristine,[21] and colchicine.[22]

Bifunctional reagents, for example succinic anhydride, are used in the synthesis of conjugates with participation of a hydroxy-group of the hapten. The anhydride has been used to obtain the conjugates of nicotine,[20] vindoline,[24] quinine,[25] and quinidine.[26] Glutaric anhydride has also been used to synthesise the vindoline conjugate.[27]

The presence of a phenolic hydroxyl in the hapten molecule permits the use of carboxymethylation in order to attach such a molecule to a protein; morphine conjugates have been obtained in this way.[28-30]

The presence of an amino-group in the hapten molecule makes it possible to obtain conjugates with the aid of the Mannich reaction or acrylic acid. The Mannich reaction has been used to synthesise the conjugates of vinblastine,[31, 32] while acrylic acid has been used to synthesise the conjugate of nor-(−)-scopolamine.[33]

The conjugation procedures described presuppose the insertion of a fairly short intervening chain (spacer) between the hapten and the protein molecule. However, in those cases where the spacer contains a chain of only 2–3 carbon atoms, it is not always possible to obtain an antiserum capable of recognising the hapten. In such cases, it is useful to increase the length of the spacer to 5–6 methylene units. An increase in the length of the spacer can be achieved, for example, by using 6-amino-n-hexanoic acid, the amino-group of which is attached to the hapten, while the carboxy-group is attached to the protein. A quinidine conjugate has been obtained in precisely this way.[26] The introduction of a spacer with a length corresponding to 4 or 5 carbon atoms is achieved using succinic or glutaric anhydride. A more rigid spacer is obtained when *trans*-cyclohexane-1,4-dicarboxylic dichloride is used, whilst a longer spacer can be secured with the aid of 4-aminohippuric acid.[34]

The nature of the spacer has a significant influence on the characteristics of the assay method. It has been established in relation to polarisation fluoroimmunoassay of methamphetamine by means of its conjugate with fluorescein that the best results are obtained when a spacer fully identical with that in the immunogenic conjugate used to obtain the antibodies is employed.[35]

The majority of conjugation methods are based on the interaction between the activated functional groups of the hapten and the amino-groups of the protein. Therefore one of the requirements which must be met by the protein is the accessibility of the ε-amino-groups of the lysine residues. Proteins such as bovine serum albumin (BSA), human serum albumin (HSA), and keyhole limpet hemocyanin (KLH) are most often used for conjugation. In certain cases, poly-L-lysin was employed for conjugation,[20, 29] but it was found that it has no appreciable advantages over the proteins listed above. It has been demonstrated empirically that it is preferable to conjugate the hapten with KLH rather than albumin in order to obtain antibodies to small compounds.[36]

In enzyme immunoassay methods, where there is conjugation with two proteins, it is important that these proteins should not be related and that the immunised animal should not have come into contact previously with these proteins.

The conjugate synthesised is usually purified by gel filtration on a column with Sephadex G-25 or by dialysis, which makes it possible to separate the unreacted low-molecular-mass components.

The molar ratio of the components of the conjugate — the hapten density — is determined in the purified conjugate. It has been established experimentally that one must avoid both very high and very low hapten densities. Thus the hapten : protein ratio for BSA should be in the range from 10 : 1 to 25 : 1, whereupon one usually obtains an antiserum of good quality. The hapten density can be frequently determined by UV or fluorescence spectroscopy, but in some cases it may be necessary to split off the hapten chemically and to determine its amount by other methods.[37]

Experiments with conjugates of low-molecular-mass compounds of plant origin hardly differ from the usual manipulations involving traditional (protein) antigens.

Since low-molecular-mass organic substances have a small and rigorously defined antigen determinant, highly specific polyclonal antibodies can be obtained successfully for them and can also be used successfully for routine assays of low-molecular-mass compounds. The monoclonal antibodies to haptens do not as a rule have special advantages and their preparation requires considerable effort and expense.

Immunochemical methods for the assay of a number of low-molecular-mass compounds (alkaloids, terpenes, vitamins, steroids, and regulators of the growth and development of plants) and biologically active compounds of plant origin such as phytohormones, cytokinins, phytoalexins, and enzymes have now been developed.

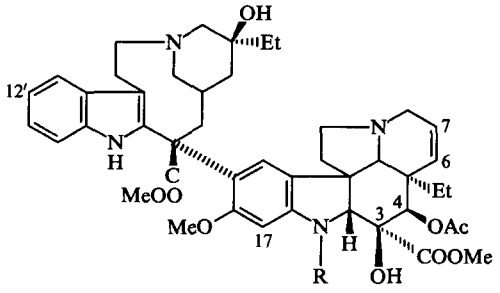
The therapeutic properties of medicinal plants are known to be largely determined precisely by low-molecular-mass compounds. The range of such compounds of plant origin, for which immunoassay methods have been developed, is being constantly expanded and these methods have now come to be fairly usual in clinical practice and are finding increasing application in plant cultivation and in the manufacture of medicinal preparations of plant origin. Immunochemical methods are used widely for the selection of the most productive plant species. Antisera to several of the low-molecular-mass biologically active substances isolated from plants and contaminating plant material are now produced industrially by a number of companies.[36, 38]

The high specificity of the antibodies makes it possible to determine individual substances in the presence of a multiplicity of other compounds or their metabolites by immunochemical methods, but in many instances it may be necessary to assay not just any one compound but a whole class of substances. The method employing mixtures of several highly specific antibodies is used for the simultaneous assay of several compounds. Thus a homogeneous polarisation fluoroimmunoassay using a mixture of antibodies to determine the majority of the classes of narcotics usually employed — amphetamines, barbiturates, and cannabinoids — has been described.[39]

III. Immune methods for the assay of alkaloids

1. Indole alkaloids of *Catharanthus roseus*

The extremely great pharmaceutical value of the alkaloids of *C. roseus*, especially vinblastine **1** and vincristine **2**, has aroused continuous interest by investigators of these alkaloids. The great demand for the alkaloids of *C. roseus*, and especially compounds **1** and **2**, has stimulated intense research designed to increase the manufacture of the alkaloids. The two main approaches to the solution of this problem — the selection of the most productive plants and the cultivation of *C. roseus* cells in fermenters — required the development of new rapid and accurate assay methods.



R = Me (1), CHO (2)

a. Vinblastine and vincristine

These bisindole alkaloids, which are minor components of the total alkaloid extract from *C. roseus*, are believed to be some of the most important biologically active substances of plant origin and are used widely in medical practice as antineoplastic agents. In view of their high activity and the presence of side effects such as neurotoxicity and myelosuppressivity, the clinical use of these alkaloids requires careful monitoring, for which immune methods are ideally suitable.

In terms of the site of the conjugation of the alkaloid molecule, the immunoassay methods for compounds **1** and **2** can be divided into several groups.[40]

Chronologically the first and most widely used procedure involves conjugation at the C(3) atom. A brief communication was published in 1975[41] concerning the development of a radioimmunoassay method for compounds **1** and **2**. Using the method for the conjugation of compound **1** to a protein, the Eli Lilly and Company Corporation (USA) began to manufacture an antiserum to the antigen obtained by converting compound **1** initially into the 3-hydrazide and then into the 3-carboxazide (4-desacetylvinblastine), which was conjugated to the protein.[42]

The antibodies to such conjugates, even monoclonal antibodies, exhibit as a rule a low specificity: cross reactions of the antibodies with derivatives of compound **1** (vindesine and its derivatives) and to compound **2** and its derivatives (leurosine and leurosidine) are observed. Polyclonal antibodies also participate in cross reactions with the monomeric alkaloids vindoline and catharanthine.

Polyclonal antibodies to the conjugates of compounds **1** and **2** via the C(3) atom have been used to develop radioimmunoassay[43] and enzyme immunoassay[32] methods for these compounds. In the radioimmunoassay method using [^{3}H]-**1** and [^{3}H]-**2**, the specimen analysed is incubated beforehand with the immune serum and is then treated with the radioactively labelled substance. This makes it possible to increase the sensitivity of the determination of the alkaloids: the concentration range in which compounds **1** and **2** can be determined is 0.2–30 ng. In the enzyme immunoassay method, compounds **1** and **2** are determined in microtitration trays the wells in which are coated by antibodies (dilution 1 : 10 000); the concentration range in which compounds **1** and **2** can be determined is 0.01–0.1 ng. However, none of these methods makes it possible to distinguish between the alkaloids **1** and **2**.

An RIA method[44] for the determination of compound **1**, also based on the use of the conjugate at the C(3) atom and used in the study of the pharmacokinetics and metabolism of compound **1** in humans, makes it possible to determine compound **1** in the concentration range 0.2–100 μg litre^{-1}. The monoclonal antibodies to the conjugates of compound **1** at the C(3) atom have been used in the development of solid-phase and liquid-phase RIA methods and an EIA method.[46] The latter permits the determination of compound **1** in the concentration range 1–100 μg litre^{-1}; the limit of the determination of compound **1** is 0.64 μg litre^{-1}. However, cross reactions with vindesine (3%), vinblastine (75%), leurosine (152%), and a number of other derivatives take place in this instance.

The conversion of compound **1** into an azide activated in the 3-position and its conjugation with the dipeptide Gly-Tyr made it possible to devise a radioimmunoassay method for vincristine and vindesine using a [^{125}I]-labelled conjugate.[47]

A conjugation method which makes it possible to determine both alkaloids and to distinguish reliably compound **1** from compound **2** was developed in 1979.[21] This method is based on the oxidation of the double bond between the C(6) and C(7) atoms with a mixture of potassium permanganate and sodium periodate.[48] Such oxidation leads to the formation of a dicarboxy-derivative, which affords a conjugate with the protein.

The antibodies formed in response to the introduction of such a conjugate into the organism are more specific: the antibodies to the conjugate of compound **1** interact with compound **2** five times more weakly than with compound **1**, whereas those to the conjugate of compound **2** interact with compound **1** 200 times more weakly than with compound **2**.

The two alkaloids **1** and **2** were converted in different ways into the 6,7-dicarboxy-derivatives, which were in their turn added to HSA. These conjugates were used to develop radioimmunoassay methods using [^{3}H]-**1** and [^{3}H]-**2**. The 'bound' and 'free' phases were separated with the aid of goat antibodies to the rabbit immunoglobulins G and the radioactivity of the 'bound' phase was measured. One of the antisera obtained proved to be specific to compound **2** and the contribution of the cross reaction with compound **1** was only 0.05%; compound **2** can be determined in the range 1–10 ng. Another antiserum was moderately selective to compound **1** and the contribution of the cross reaction with compound **2** was 25%; the range for the determination of compound **1** is 1–15 ng and the limit in its determination is 0.4 pg litre^{-1}.[21] These antisera can be used to select plants in terms of their content of compounds **1** and **2**.

A further increase in the sensitivity and selectivity of the determination of compounds **1** and **2** was achieved using an enzyme immunoassay method employing compound **1** attached by the Mannich reaction to BSA via the C(17) atom.[31, 32] The C(17) atom of the aromatic ring, which is close to the carbon atom carrying the methoxy-group, proves to be activated and is capable of interacting with formaldehyde.

The antibodies to the conjugate via the C(17) atom were used to develop a method for the radioimmunoassay of compound **1**. The method makes it possible to determine compound **1** in the plasma in the concentration range from 1 to 200 to 300 μg litre^{-1}.[31] An enzyme method for the immunoassay of compound **1** with the aid of monoclonal antibodies was developed later. This method made it possible to determine compound **1** in the concentration range 1–100 μg litre^{-1}; the limit in the determination of compound **1** is 0.54 μg ml^{-1}.[32]

The monoclonal antibodies to the conjugate via the C(17) atom are somewhat more specific than the antibodies to the conjugate via the C(3) atom, but appreciable contributions of the cross reactions with the derivatives of compound **1** (vindesine) compound **2** and its derivatives (leurosine, and leurosidine) have been noted for them also.[32]

Studies using the method involving the conjugation of compound **2** via the C(12′) atom of the aromatic ring of the molecule have been published in recent years.[49-51] The antibodies to the conjugate via the C(12′) atom are highly selective and there are virtually no cross reactions with compound **1**. For this purpose, *p*-aminobenzoic acid was added to the C(12′) atom [corresponding to the C(10) atom according to the biogenetic numbering] of compound **2** after nitration and the resulting derivative was attached to the protein via the amino-groups by the carbodiimide method.[49]

This conjugation procedure makes it possible to distinguish more reliably compounds **1** and **2**: the antibodies to the conjugate of compound **2** interact with compound **1** and vindoline 1500 times more weakly than with compound **2**.[50, 51]

The antiserum to this conjugate was used to develop a method for the radioimmunoassay of compound **2**.[51] The range of the determination of the latter is 0.1 – 100 ng; the contributions of the cross reactions with compound **1** and vindoline are 0.03% and 0.09% respectively. A method for the enzyme immunoassay of compound **2** was developed on the basis of the same conjugate;[50] the range of the determination is 5 – 500 pg.

A study in which compound **1** was attached to the protein with the aid of glutaraldehyde[53] was published in 1989.[52] The use of this conjugate leads to the formation of an antiserum, which recognises mainly compound **1**. This made it possible to develop a method for the radioimmunoassay of compound **1** in the range 0.5 – 10 ng ml^{-1}. The contributions of the cross reactions with compound **2** and leurosine are 30% and 0.23% respectively and that of the cross reactions with all the remaining alkaloids of *C. roseus* is less than 0.1%.

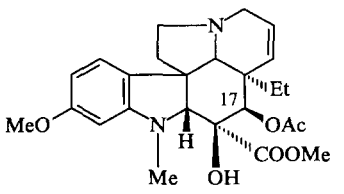
This conjugate was used in the development of a method for the radioimmunoassay of compound **1** in *C. roseus* tissue cultures.[52]

It is noteworthy that almost all the methods for the immunoassay of compounds **1** and **2** were developed and used to determine the content of these alkaloids in the blood sera of patients injected intraveneously with the preparations **1** and **2**[31, 43, 45, 50] and for the investigation of the pharmacokinetics and metabolism of these alkaloids.[32, 44, 54] Immunoassay methods have not so far found extensive application in the determination of the contents of compounds **1** and **2** in plants. Only radioimmunoassay[21] and enzyme immunoassay[50] methods have been used for the quantitative assay of the alkaloids in various parts of the *C. roseus* plant. It was established that the maximum concentration of the alkaloids is detected in the flowers, but, owing to the small absolute mass of the flowers, almost the total amount of the alkaloids is contained in the leaves.[21] The content of compound **2** in the extracts from the leaves of *C. roseus* was determined by an enzyme immunoassay method.[50] The total amount of the dimeric indole alkaloids in *C. roseus* was likewise determined by an enzyme immunoassay method.[46] The immuno methods are used somewhat more widely in the quantitative assay of compounds **1** and **2** in *C. roseus* cell cultures.[52, 55-57]

The high cost and pharmaceutical value of compounds **1** and **2** have led to the intensification of the development of methods for the immunoassay of these substances. This led to the appearance in the world market of primary antisera to compounds **1** and **2** manufactured by companies such as Bioanalysis Ltd and Guildhay Antisera (Great Britain) and Eli Lilly and Company.

b. Vindoline

Vindoline **3** is an indole alkaloid which is a biogenetic precursor of compounds **1** and **2** and can be used for the chemical synthesis of compound **1**.[58] In the development of a method for the immunoassay of compound **3**, 17-desacetylvindoline was initially activated by succinic anhydride and the resulting hemisuccinate was then attached to BSA by the carbodiimide method.[27] The antiserum obtained with the aid of this conjugate was used in the radioimmunoassay of compound **3**; the range of its determination is 0.2 – 45 ng. The conjugate of 17-desacetylvindoline with BSA was also synthesised with the aid of succinic anhydride.[24] This conjugate was used in the development of a method for the radioimmunoassay of compound **3**, but it proved to be less sensitive: the range of the determination of compound **3** is 10 to 1000 ng.



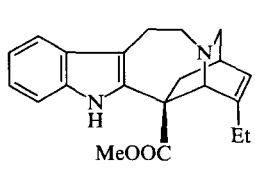
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The use of the antiserum to the conjugate of compound **1** with BSA, obtained by reaction via the C(12′) atom, made it possible to develop methods for the enzyme immunoassay and radioimmunoassay of compound **3**.[59, 60] The range in which compound **3** can be determined by enzyme immunoassay is 5 to 500 pg.

These methods were used to determine the content of compound **3** in various parts of the *C. roseus* plant[27] and in *C. roseus* cell cultures.[27, 59, 61]

c. Catharanthine

Like compound **3**, catharanthine **4** is a biogenetic precursor of compounds **1** and **2** and constitutes, as it were, the 'upper' half of the molecules of the dimeric alkaloids and, like compound **3**, can be used for the chemical synthesis of the dimeric alkaloids. A method for the radioimmunoassay of compound **4** in the range 0.02 – 20 ng has been developed.[61] In order to obtain an antiserum, rabbits were immunised with a conjugate of catharanthic acid with HSA. [^{3}H]-**4** was used as the radioactive ligand. The limit in the radioimmunoassay of compound **4** is 66 pg. Cross reactions with catharanthic acid and the dihydro- and carboxy-derivatives of compound **4** have been noted.

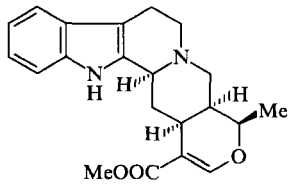


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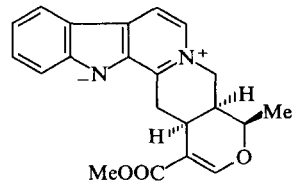
The radioimmunoassay has been used to determine compound **4** in *C. roseus*.[62]

d. Ajmalicine and serpentine

Ajmalicine **5** is one of the principal alkaloids isolated from plants of the *Catharanthus* and *Rauwolfia* species. It is used widely in the treatment of the blood circulation system — in particular in the pathology of the vessels in the brain. Serpentine **6**, which is an alkaloid related to ajmalicine, is not used as a medicinal drug but can serve as a source of compound **5**.



5



6

For the effective production of compound **5**, it is important to select plants in terms of the maximum contents of compounds **5** and **6**. Antisera to each of these alkaloids were therefore

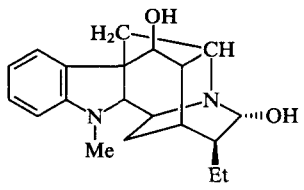
obtained.[19] For this purpose, ajmalinic acid was obtained by the alkaline hydrolysis of compound **5** and was attached to HSA by a modified mixed anhydride method. The resulting conjugate was used to produce an antiserum; the antiserum proved to be specific to compound **5** and was insensitive to compound **6**. The radioimmunoassay, developed using this antiserum makes it possible to determine compound **5** in the range 2–50 ng.[19]

Serpentinic acid, obtained by the alkaline hydrolysis of compound **6**, was conjugated with HSA via the *N*-hydroxysuccinimide ester modified by a carbodiimide method. The antiserum to this conjugate proved specific to compound **6** and insensitive to compound **5**. The radioimmunoassay makes it possible to detect compound **6** in the range 2–50 ng.[19]

Both these methods were used in the study of the distribution of compounds **5** and **6** in plant tissues. It was shown that, in contrast to compound **3**, the maximum concentration of both alkaloids is found in the plant roots immediately under the surface of the soil, the concentration of compound **6** being approximately five times higher than that of compound **5**. Radioimmunoassay was used in selection,[19] which made it possible to select a population of plants with an increased overall content of alkaloids and an increased **5**/**6** ratio, and also in the determination of these alkaloids in the cultivation of *C. roseus* cells by the suspension method.[62] The same methods have been used in the study of the biosynthesis of compound **5**.[63]

e. Ajmaline

In order to obtain an antiserum specific to the alkaloid ajmaline **7**, the hemisuccinate of compound **7** was conjugated with BSA and rabbits were immunised with this conjugate.[64] The resulting antiserum made it possible to develop a radioimmunoassay for compound **7**. [^{3}H]-**7** was used as the radioactive ligand. The range in which compound **7** can be determined is 0.05–20 ng. Appreciable cross reactions with 17-chloroacetylajmaline, sandwicine, and isoajmaline have been noted.



7

The method developed has been used to determine the content of compound **7** in the plants of the *Rauwolfia* family.[64]

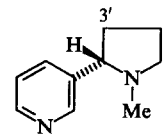
2. Tobacco alkaloids

One of the best known alkaloids of tobacco (*Nicotiana tabacum*, *Nicotiana rustica*, etc.) is nicotine **8**. At the present time compound **8** is isolated from plants mainly in order to oxidise it to nicotinic acid and to synthesise preparations based on it, while the alkaloid itself has found a limited application as an insecticide and an ectoparasitocide in veterinary practice.

In 1973 a radioimmunoassay was developed for compound **8** and its principal metabolite in the organism—cotinine **9**.[20]

a. Nicotine

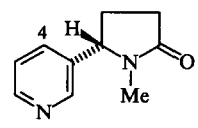
In order to synthesise an antigen for the assay of compound **8**, 3′-hydroxymethylnicotine, synthesised by the procedure described by Cushman and Castagnoli,[65] was activated with succinic anhydride and *trans*-3′-succinylmethyl niccotine was obtained and attached to HSA, KLH, and poly-L-lysine by the carbodiimide method. [^{3}H]-**8** was obtained by the catalytic ^{3}H exchange involving the pyridine ring. In the radioimmunoassay, the 'bound' and 'free' phases were separated by immunoprecipitation using goat antibodies to the rabbit Ig G and the radioactivity of the 'bound' phase was measured. The method makes it possible to determine compound **8** in the range 0.35 to 7 ng and an appreciable cross reaction has been observed only with *dl*-amino-**8** (2.4%). Cotinine is not detected if its content in the sample does not exceed 50 ng.[20]



8

b. Cotinine

In the synthesis of an immunogen for the assay of cotinine **9**, *trans*-4-carboxy-**9** was conjugated by the carbodiimides method with HSA, KLH, and poly-L-lysine. Radioactive ^{125}I was introduced into *N*-(4-hydroxyphenylethyl)-*trans*-carboxamide derived from compound **9**. The assay was carried out as for compound **8**. The sensitivity of the method corresponds to 0.01–1 ng. An appreciable cross reaction has been observed only for *dl*-desmethyl-**9** (0.9%); at a level below 70 ng, compound **8** is not detected.[20]

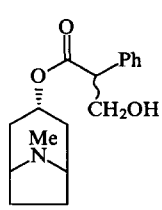


9

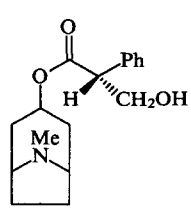
These methods have been used to investigate the conversion of compound **8** into compound **9** by extracts from rabbit liver. It was shown that compound **8** is not fully oxidised: after 10 h, only 37% of compound **8** is converted into compound **9**. These methods were also used to analyse the conversion of compound **8** into compound **9** *in vitro*, using extracts from cultivated liver cells, and for the determination of the content of these alkaloids in the biological liquids of smokers. It was established that the principal metabolite of compound **8** present in the blood serum and urine of smokers is compound **9**. Its content in the blood serum and urine falls after smoking has stopped. Compound **9** was not detected in the biological liquids of non-smokers.[20] The same method has been used to determine the content of nicotine and its metabolites in hair.[66] The method has not been used to determine compound **8** in plant tissues.

3. Tropane alkaloids

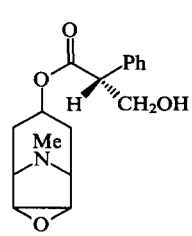
The structures of these alkaloids are based on the tropane skeleton. The most familiar of these are atropine **10**, hyoscyamine **11**, and scopolamine **12**. The alkaloids of the cocaine group, which will be examined below, can also be included with them. Antropine constitutes the racemic form of hyoscyamine formed when the alkaloids are isolated from plants.



10



11



12

Scopolamine, produced by plants of the *Datura* and *Duboisia* (*Solanaceae*) species, is used as a parasympatolytic, antiemetic, and anticholinergic agent. Being a typical agent blocking *m*-cholinoreceptors, it resembles compound **10** as regards its physiological activity, but its influence on the central nervous

system is much stronger and it is frequently used as a calming agent in psychiatry, in narcology, and in sea sickness. Together with the alkaloid **12**, plant tissues usually contain large amounts of compound **11** – the biogenetic precursor of compound **12**.

In the synthesis of the immunogenic conjugate, compound **12** was attached to HSA via the hydroxy-group with the aid of glutaric anhydride, but an active antiserum to this conjugate was not obtained.[33] A satisfactory antigen was synthesised by the reaction of nor-(−)-**12** with acrylic acid and subsequent attachment of the resulting derivative to HSA by the mixed anhydride method or the carbodiimide method. The antiserum to this conjugate, specific to compound **12** and nor-**12**, made it possible to develop a method for the radioimmunoassay of compound **12** in the range 0.5–50 ng. The contribution of the cross reaction with compound **12** is only 0.8%.

The method has been used to investigate the distribution of compound **12** in the tissues of *D. innoxia* and *D. sanguinea*. The maximum concentration of compound **12** was detected in unripe seeds, but, owing to the small absolute amount of the seeds, almost the entire alkaloid **12** is contained in the leaves. A similar method has been used to assay compound **12** in *Datura* tissue cultures.[67]

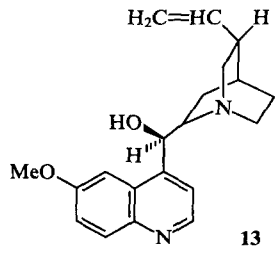
It was later reported[68] that an immunoassay had been developed using the rabbit antiserum to the conjugate of tropic acid with BSA. The method permits the simultaneous determination of the principal tropane alkaloids **10**–**12**. The detection limit of compound **10** is 0.1 ng. In the determination of compounds **11** and **12**, the latter are first racemised.

4. Quinoline alkaloids

Certain plant species of the genus *Cinchona* (*Rubiaceae*) produce the isomeric alkaloids quinine **13** and quinidine **14**. From the pharmacological point of view, compound **13** is an antibiotic. It is effective against the gametocytic forms of *Plasmodium vivax* and *Plasmodium malariae* in erythrocytes, the mechanism of its biological activity, which consists in the selective suppression of the replication of DNA and the transcription of RNA, being typical for antibiotics. Quinine is widely used as an antimalarial agent and as a bitter and has also found limited application in the treatment of certain cardiac affections and in midwifery. Quinidine, a stereoisomer of compound **13**, is a powerful antiarrhythmic agent.

a. Quinine

Activation of compound **13** with succinic anhydride and its attachment to BSA by the mixed anhydride method afford an immunogenic conjugate, which makes it possible to obtain an antiserum specific to compound **13**. A radioimmunoassay[25, 26] and an enzyme immunoassay[26] were developed on the basis of the antiserum and permitted the determination of compound **13** in the ranges 1–50 and 0.1–10 ng respectively. The detection limit of compound **13** is 10 pg for the enzyme immunoassay and 50 pg for the radioimmunoassay.[26]

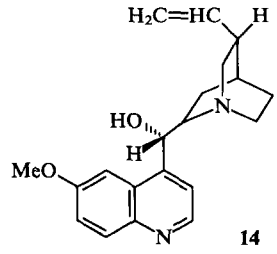


13

The enzyme immunoassay method was used to determine compound **13** in specimens of human biological liquids.[69]

b. Quinidine

There exist at present analytical sets for the immunoassay of compound **14**. The Beckman Company (USA) has developed an analytical set for nephelometric immunoassay, which makes it possible to determine compound **14** and a number of its physiologically active metabolites in mammalian organisms.[70] The Syva Corporation manufactures an analytical set for the immunoassay of compound **14**.[71] Both methods are designed to use special equipment, require a large amount of antiserum, and their sensitivity is fairly low (1–10 ng ml^{-1}). However, the complete automation of the assay process makes them very convenient for clinical application.



14

The immunoassay method developed by the Syva Corporation has been compared with the method using reversed-phase HPLC combined with detection by the fluorescence method.[72] It has been established that, in the determination of the content of compound **14** in the blood sera of patients receiving it, the enzyme immunoassay method yields values higher by a factor of 1.2 than the chromatographic method. An automatic system has been developed for the immunoassay with a fluorescence-labelled ligand, which makes it possible to determine compound **14** in the presence of other substances.[73]

Radioimmunoassay and enzyme immunoassay methods were developed in 1985 using the antiserum to the conjugate of 9-hemisucinylquinidine attached to BSA via 6-amino-n-hexanoic acid.[26] The ranges in which compound **14** can be determined by the radioimmunoassay and the enzyme immunoassay methods are 1–1000 ng and 0.1–10 ng. The radioimmunoassay method has been used for the screening of the clonal cultures of *C. ledgeriana* in analysis designed to determine compound **13**.[25]

The immune method based on the measurement of the degree of polarisation of fluorescence has been used to investigate the pharmacokinetics of compound **14**.[74] The rapid combined autofiltration–immunoenzyme and dialysis–immunoenzyme methods for the assay of compound **14** in the blood sera of patients yielded results which agreed well with those obtained by the autofiltration–liquid- chromatographic and dialysis–liquid-chromatographic methods.[75]

The immunoenzyme and immunofluorescence methods for the assay of compound **14** have come to be fairly widely used, principally in clinical practice.[76–78] An automatic system 'optimate', which makes it possible to assay compound **14** by the immunofluorescence method, has been developed and is being actively employed.[73]

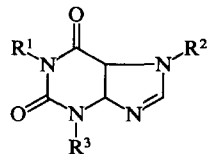
5. Purine alkaloids

This group includes derivatives of xanthine ($R^1 = R^2 = R^3 = H$): caffeine **15** ($R^1 = R^2 = R^3 = Me$), theophylline **16** ($R^1 = R^3 = Me$, $R^2 = H$), and theobromine **17** ($R^1 = H$, $R^2 = R^3 = Me$). Caffeine is present in tea leaves and coffee beans, theobromine was first detected in cocoa beans, and theophylline was detected in tea.

The mechanism of the physiological activity of these alkaloids involves the inhibition of phosphodiesterase and the accumulation of an intracellular mediator—cyclic adenosine-3′,5′-monophosphate (cAMP).

Two groups of methods are used for the quantitative assay of xanthine derivatives: the immune methods and methods based on HPLC.[79]

The presence in the molecules of these compounds of a large number of functional groups makes it possible to obtain fairly readily immunogenic conjugates of these alkaloids with proteins.



15–17

a. Caffeine

Caffeine is a typical psychostimulator. It also excites cardiac activity, dilates coronary blood vessels, and intensifies the respiratory activity and diuresis.

Immunoassay methods for the determination of compound **15** are used nowadays only in medical practice — in particular, the method for the enzyme immunoassay of compound **15** in the serum is used in pediatrics.[80]

Results of assays of compound **15** in serum and plasma by the immunoenzyme and HPLC methods have been compared and it has been shown that they are virtually identical.[80, 81]

b. Theophylline

Theophylline exhibits a physiological activity similar to that of compound **15**, but its broncholytic and diuretic activities predominate.

The fairly wide use of compound **16** as a medicinal drug has promoted the development of a large number of immunoassay methods for this alkaloid. The immunoenzyme,[82–86] immunofluorescence,[73, 87–90] immunochromatographic,[91] and immunoturbidimetric[92, 93] methods, the immunochromatographic methods using apoenzyme reagent strips,[94–98] and an immunoenzyme method employing an enzyme encapsulated in liposomes[99] are most widely used nowadays. The 'Optimate' system, based on the immunofluorescence method, is used for the automatic assay of compound **16**.[73]

These methods have been used in the study of the pharmacokinetics of compound **16**,[100–102] in the determination of the content of compound **16** in dry blood spots,[83, 84] in whole blood,[97] in urine,[103] in saliva,[88] in blood serum,[82, 83, 93–95, 104–106] and in the plasma.[89–91, 107–111]

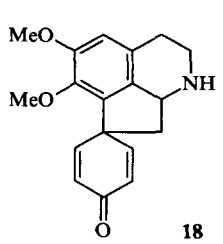
The influence of substances usually encountered in the specimens analysed on the results of the determination of compound **16** has been investigated.[108, 112–115]

Various immunoassay methods and the method based on the HPLC have been compared.[92, 98, 101, 104–107, 109–111] It has been established that the immunoenzyme method, the immune method based on the measurement of the degree of polarisation of fluorescence, and the method based on HPLC are best in terms of the accuracy and reproducibility of the results.[103, 109]

6. Proaporphine alkaloids

The structure of these alkaloids is based on that of aporphine.

Stepharine **18** is an alkaloid isolated from *Stephania glabra*. It exhibits a cholinesterase activity and is used in the treatment of diseases of the peripheral nervous system, myopathy, lateral amyotrophic sclerosis, and paresis of the facial nerve.

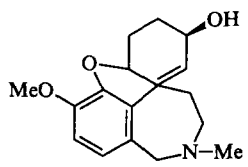


18

In order to obtain antibodies, compound **18** was treated with succinic anhydride and then with *N*-hydroxysuccinimide and *N,N'*-dicyclohexylcarbodiimide and was conjugated with BSA. The antibodies obtained with the aid of this conjugate were used in the development of a solid-phase enzyme immunoassay method with a sensitivity of 10 pg ml^{-1}. The method was used to assay compound **18** in a plant tissue culture and also for its determination in the blood of patients.[116]

7. Alkaloids of the physostigmine group

Galanthamine **19** is an alkaloid isolated from the tubers of the snowdrops *Galanthus woronowii* and *Galanthus nivalis*. The mechanism of its physiological activity is associated with the intensification of the influence of acetylcholine on the *n*-cholinergic synapses as a result of the inhibition of cholinesterase. It is used in medical practice to reduce the pressure within the eyeball and also for the treatment of certain nervous diseases and for the intensification of peristalsis on paresis of the intestine.

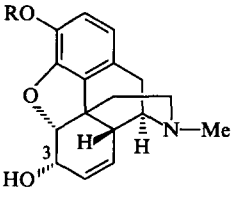


19

There exists a specific method for the radioimmunoassay of compound **19**. In order to obtain an immunogenic conjugate, the alkaloid **19** was treated with succinic anhydride and galanthamine 2-*O*-hemisuccinate was obtained and conjugated with BSA with the aid of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide. The method makes it possible to determine compound **19** in the range 0.5–100 ng.[117]

8. The poppy alkaloids

This group includes morphine **20**, codeine **21**, narcotine, papaverine **22**, thebaine, and about 20 other alkaloids.



R = H (**20**), Me (**21**)

a. Morphine

Morphine is the main alkaloid isolated together with 20 other alkaloids from the opium poppy (*Papaver somniferum*). Morphine belongs to the class of analgesic substances — narcotic analgesics. It also exhibits sedative and soporific activities, stimulates the smooth muscle, and at high concentrations induces vomiting and constipation, hinders diuresis, and inhibits the thermal regulation and respiration centres. It has been established that compound **20** influences specific receptors in the cortex of the cerebral hemispheres; it is widely used as an analgesic narcotic.

b. Codeine

Codeine also forms part of the composition of *P. somniferum*. It exhibits a weak narcotic activity and is widely used as an analgesic and also as an anti-cough preparation.

In view of the difficulties in the cross cultivation and selection of strains of *P. somniferum*, it was desirable to have a rapid method for the assay of compound **20**, which led at the beginning of the 1970s to the development of methods for the immunoassay of compound **20**.

Radioimmunoassay methods[28, 29, 118, 119] and methods based on the inhibition of haemagglutination[30, 120] were developed for the determination of compounds **20** and **21**. In all cases the immunogenic conjugate was obtained by the carboxymethylation of compound **20** at the hydroxy-group in the 3-position and by the attachment of the resulting derivative to the protein, as well as conjugation by the carbodiimide method. The antiserum to this conjugate was isolated from rabbit or guinea pig blood.

The most sensitive and specific method proved to be the radioimmunoassay employing the copolymer of L-Glu, L-Lys, L-Ala, and L-Tyr, to the ε-amino-groups of the lysine residues of which 3-*O*-carboxymethylmorphine had been attached and which had been [^{125}I]-labelled in the tyrosine residues, as well as the antiserum from the guinea pig blood. The range in which compound **20** can be determined is 0.05–5 ng. A disadvantage of this method[29] is the impossibility of distinguishing between compounds **20** and **21**. The method has been used to determine the lifetime of compound **20** in the blood[118] and also in the analysis of blood serum specimens taken in the case of the so called 'sudden death'. It has been shown that the results of the radioimmunoassay agree well with the results obtained by chromatographic methods.[29]

A homogeneous method for the enzyme immunoassay of compound **20**, based on the use a of conjugate of morphine with lysozyme and antiopiate antibodies, was developed at the beginning of the 1970s.[121, 122] This method makes it possible to determine compound **20** in the concentration range 0.5 to 3 μg ml^{-1}. Subsequently lysozyme was replaced by other enzymes (malate dehydrogenase and glucose-6-phosphate dehydrogenase), which made it possible to increase the sensitivity by a factor of 10.[123–125]

The antibodies to compound **20** were used to develop a method and a reagent set for the simultaneous automatic enzyme immunoassay of a number of narcotic preparations in urine microspecimens.[126] The concentration limit for the detection of compound **20** is 0.3 mg ml^{-1}.

The enzyme immunoassay method has been used for the determination of compound **20** in specimens of the blood of persons who died as a result of the consumption of heroin.[127]

A brief communication about the development of methods for the radioimmunoassay of (*S*)- and (*R*)-reticuline, salutaridine, thebaine, and compounds **20** and **21** appeared in 1984. All the antisera obtained were highly specific and made it possible to determine the above substances in the range 0.01–10 ng.[128] These methods make it possible to investigate the pathways leading to the biosynthesis of the above alkaloids and to select plants or cell cultures in terms of the productivity of the biosynthesis of these alkaloids.

Methods for the immunoassay of compound **20** are used mainly in clinical practice.[129] An immunoassay method involving the use of indicator strips has been used successfully to detect compound **20** in urine.[130]

A method based on latex agglutination has found extensive application in the screening assay of opiates.[131]

A heterogeneous enzyme immunoassay method is also used for the detection of opiates.[132] Competitive solid-phase enzyme immunoassay of opiates makes it possible to attain a high sensitivity — up to 0.25 ng ml^{-1}. When the method is used in its qualitative version without instrumental enhancement, the sensitivity of the determination is 300 ng ml^{-1}, which is comparable to the sensitivity of the homogeneous enzyme immunoassay.

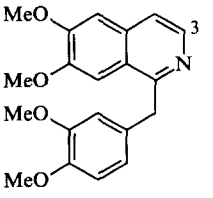
Information concerning the development of highly sensitive immunofluorescence methods for the assay of compound **20** in urine was published subsequently.[133, 134]

The Hofmann La Roche Company (Switzerland) produced a reagent set for a qualitative visual assay of opiates with a sensitivity of 300 ng ml^{-1}.[135]

Immunoassay methods are used for the determination of opiates in serum, urine, and saliva specimens as well as other human biological liquids.[135] There exists a highly sensitive method which makes it possible to determine compound **20** in hair;[136] the sensitivity reaches 0.2 ng ml^{-1} under these conditions.

c. Papaverine

Papaverine **22** is also one of the most valuable alkaloids isolated from the poppy plant. It exhibits spasmolytic and moderate vasodilating activities and increases the consumption of oxygen by the myocardium. Papaverine is administered in the hypertonic state, in stenocarditis, and in the treatment of the spasms of the coronary and brain blood vessels, the smooth muscle of the abdominal cavity, and other diseases.



22

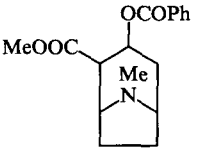
A conjugate of synthetic 3-aminopapaverine has been used to obtain antibodies to compound **22**.[137] For this purpose, 3-aminopapaverine was treated with glutaric anhydride and converted into 1-(3,4-dimethoxybenzyl)-3-*N*-(4-carboxybutyryl)-amino-6,7-dimethoxypapaverine, which was attached to BSA with the aid of *N*-hydroxysuccinimide and *NN'*-dicyclohexylcarbodiimide. A solid-phase enzyme method for the immunoassay of compound **22** was developed on the basis of the antibodies obtained.[138] Its sensitivity is 10^{-6} g ml^{-1}.

9. Alkaloids of the cocaine group

These alkaloids are present in the leaves of the coca plant (*Erythroxylon coca*). Their structure is based on the bicyclic tropane skeleton and in terms of this feature they can be included among tropane alkaloids.

As in the case of the purine alkaloids, the large number of functional groups in the molecules of these alkaloids makes it possible to obtain readily immunogenic conjugates with proteins.

Cocaine **23** exhibits a powerful local analgesic activity and is a local anaesthetic. It has a paralysing effect on the parasympathetic nervous system.



23

There exists at present a fluorescence-polarisation immunoassay method which makes it possible to determine compound **23** in urine at a concentration of 1 mg ml^{-1} and above.[40]

As for other narcotics, methods for the immunoassay of compound **23** and its metabolites are used virtually only in medical practice.[139]

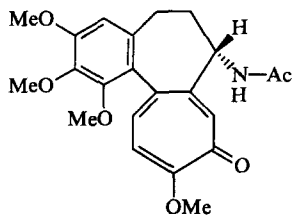
The attempts to simplify and accelerate the assay of narcotics have led to the appearance of methods involving the simultaneous assay of a broad spectrum of narcotics. Mixtures of antibodies to each substance or group of substances are used in these procedures. Among them, one may note the fluorescence-polarisation method which permits the simultaneous determination of the metabolites of compound **23**, amphetamine, and certain barbiturates in urine,[39] as well as an immunoenzyme method for the simultaneous assay of amphetamine, quinalbarbitone, oxazepam, benzoylecgonine, methadone, methaqualone, compound **20**, phencyclidine, phenytoin, propoxyphene, and nortriptyline in urine.[126]

Methods for the determination of narcotics proposed by the Syva Corporation and based on homogeneous immunoassay have come to be widely used in narcological practice.[98, 121, 124, 136, 140] Subsequently these methods have been automated.[140]

10. Alkaloids of the colchicine group

Colchicine **24** and colchamine are present together with other alkaloids in the pods of plants of the *Colchicum* and *Merendera* genera.

Colchicine is widely used in selection procedures as an agent which induces the doubling of the number of chromosomes and the formation of polyploid forms of plants and also in the study of the cytoskeleton of plants. Being a poison with a neuroparalytic activity, it is used at the same time for the treatment of the cancer of the gullet and skin and as an anticonvulsant and is in addition the best drug for the treatment of Mediterranean fever available at present.



24

A method for the radioimmunoassay of compound **24** was developed in 1975 using a conjugate of *N*-desacetylthiocolchicine, attached by the carbodiimide method to BSA, and [^{3}H]-**24**. The method makes it possible to determine compound **24** in the range 5–50 ng.[22] Cross reactions with *N*-desacetylthiocolchicine and *N*-desacetyl-*N*-colchicine (8%) have been noted. A method has been developed for the assay of compound **24** in human blood serum; it has not been used for the determination of compound **24** in plants.

A similar method, based on the use of monoclonal antibodies, which made it possible to increase appreciably the specificity of the assay, was developed subsequently.[141]

IV. Conclusion

Immunochemical methods for the assay of alkaloids are becoming increasingly popular. They are superior to chromatographic methods as regards sensitivity and speed of preparation of the analytical samples and are scarcely inferior to them as regards accuracy and reproducibility.

In the development of methods for the immunoassay of alkaloids, it is possible in most cases to obviate the difficulties associated with the preparation of immunogenic conjugates of the alkaloids with proteins and to obtain fairly specific antisera.

The methods for the radioimmunoassay of alkaloids, which were in the forefront in the 1960s–1970s, yielded the leading position in the 1980s to the enzyme immunoassay method; immunofluroescence methods are beginning to be used with increasing frequency.

The widest field in which immunoassay methods for alkaloids are used involves the measurement of their concentrations in the physiological fluids of patients receiving alkaloids preparations. This is associated both with the necessity for the rapid and accurate monitoring of the course of treatment and with the study of the pharmacokinetics and metabolism of alkaloids.

Rapid and accurate methods for the assay of alkaloids exhibiting narcotic properties are important from both medical and criminological points of view. Here too immunoassay methods are being employed to an increasing extent.

Since in many instances alkaloids are still being obtained from plant raw material, it is frequently necessary to know their content in such material. However, the lack in most countries of norms for the content of biologically active substances in plant raw material is an inhibiting factor in the development of immunoassay methods for plants. However, in a number of instances immunoassay methods have been used in studies on the biosynthesis of certain alkaloids in plants.

The deficiencies of the traditional procedure for the isolation of alkaloids from plant raw material—the dependence of the content of the alkaloids on the climatic and weather conditions, the difficulties in the collection of the plants (for plants growing under wild conditions), and the need for fertile soil (for cultivated plants)—have led to the active development at the present time of biotechnological methods for the synthesis of alkaloids in plant cell and tissue cultures. It is in the field of the monitoring of the content of alkaloids in such cultures that the immunoassay methods are being increasingly widely used and are replacing the chromatographic methods traditionally employed for such purposes.

References

1. V Marko *Farm. Obz.* **55** 37 (1986)
2. S D Varfolomeev, S V Zaitsev, A T Mevkh *Fiziko-khimicheskie Issledovaniya Molekulyarnykh Mekhanizmov Deistviya Fiziologicheski Aktivnykh Soedinenii. Itogi Nauki i Tekhniki. Ser. Bioorganicheskaya Khimiya* (Physicochemical Studies on the Molecular Mechanisms of the Activity of Physiologically Active Compounds. Advances in Science and Engineering. The Bioorganic Chemistry Series) (Moscow: Izd. VINITI, 1985) Vol. 3, p. 116
3. E L Manchester, E Geisbrecht, S J Soldin *Ther. Drug Monit.* **9** 61 (1987)
4. R V Petrov, I V Berezin *Zh. Vses. Khim. O-va im. D. I. Mendeleeva* **27** 362 (1982)
5. B B Dzantiev, A M Egorov *Zh. Vses. Khim. O-va im. D. I. Mendeleeva* **27** 442 (1982)
6. A M Egorov *Zh. Vses. Khim. O-va im. D. I. Mendeleeva* **33** 494 (1988)
7. S A Eremin *Zh. Vses. Khim. O-va im. D. I. Mendeleeva* **34** 46 (1989)
8. H Friemel (Ed.) *Immunological Methods* (Translated into Russian) (Moscow: Meditsina, 1987)
9. T T Ngo, H M Lenhoff (Eds) *Immunoenzymic Analysis* (Translated into Russian) (Moscow: Mir, 1988)
10. A M Egorov (Ed.) *Neizotopnye Metody Immunoanaliza. Itogi Nauki i Tekhniki. Ser. Biotekhnologiya* (Non-isotopic Immunoassay Methods. Advances in Science and Engineering. The Biotechnology Series) (Moscow: Izd. VINITI, 1987) Vol. 3
11. D M Kemeny, S J Challacombe (Eds) *ELISA and Other Solid Phase Immunoassays. Theoretical and Practical Aspects* (New York: Wiley, 1988)
12. B A Morris, M N Clifford (Eds) *Immunoassays in Food Analysis* (London: Elsevier, 1985)
13. H F Linskens, J F Jackson (Eds) *Immunology in Plant Sciences* (Berlin: Springer, 1986)
14. T Braun, A Klein, S Zsindely, W S Lyon *Trends Anal. Chem.* **11** 5 (1992)
15. C C Harris, R H Yolken, H Krokan, I C Hsu *Proc. Natl. Acad. Sci. USA* **76** 5336 (1979)
16. K Landsteiner *The Specificity of Serological Reactions* (Cambridge: Harvard University Press, 1946)
17. R J Robins, M R A Morgan, M J C Rhodes, J M Furze, in *Immunoassays in Food Analysis* (Eds B A Morris, M N Clifford) (London: Elsevier, 1985) p. 1977
18. B F Erlanger, O F Borek, S M Beiser, S Leiberman *J. Biol. Chem.* **234** 1090 (1959)
19. H Arens, J Stockigt, E W Weiler, M H Zenk *Planta Med.* **34** 37 (1978)
20. J J Langone, H B Gjika, H Van Vunakis *Biochemistry* **12** 5025 (1973)
21. J J Langone, M R D'Onofrio, H Van Vunakis *Anal. Biochem.* **95** 214 (1979)
22. C Boudene, F Duprey, C Bohuon *Biochem. J.* **151** 413 (1975)
23. H Meisner, P Meisner *Arch. Biochem. Biophys.* **208** 146 (1981)
24. J P Kutney, L S L Choi, B R Worth *Phytochemistry* **19** 2083 (1980)

25. R J Robins, A J Webb, M J C Rhodes, J M Furze *Planta Med.* **50** 235 (1984)
26. M R A Morgan, S Bramham, A J Webb, R J Robins, M J C Rhodes *Planta Med.* **51** 237 (1985)
27. P Westekemper, U Wieczorek, F Gueritte, N Langlois, Y Langlois, P Potier, M H Zenk *Planta Med.* **39** 24 (1980)
28. S Spector, C W Parker *Science* **168** 1347 (1970)
29. H Van Vunakis, E Wasserman, L Levine *J. Pharmacol. Exp. Ther.* **180** 514 (1972)
30. F L Adler, C-T Liu *J. Immunol.* **106** 1684 (1971)
31. J D Teale, J M Clough, V Marks *Br. J. Clin. Pharmacol.* **4** 169 (1977)
32. M P Hacker, J P Dank, W B Ershler *Cancer Res.* **44** 478 (1984)
33. E W Weiler, J Stockigt, M H Zenk *Phytochemistry* **20** 2009 (1981)
34. P S Jourdan, R L Mansell, E W Weiler *Planta Med.* **44** 82 (1982)
35. S A Eremin *Ther. Drug Monit.* **10** 400 (1988)
36. J Marrero *J. Med. Technol.* **1** 47 (1984)
37. M R A Morgan, R McNerney, J A Matthew, D T Coxon, H W-S Chan *J. Sci. Food Agric.* **34** 593 (1983)
38. L Fukal, J Kas *Trends Anal. Chem.* **8** 112 (1989)
39. D L Colbert, M Childerstone *Clin. Chem.* **33** 1921 (1987)
40. S K Volkov *Lekarstvennoe Rastenievodstvo. Obzornaya Informatsiya* (Medicinal Plant Cultivation. Review Information) (Moscow: Izd. VNIISENTI NPO "Medbioekonomika", 1990)
41. M A Root, K Gerzon, R W Dyke, in *Federation of Analytical Chemistry and Spectroscopy Societies, 2nd National Meeting, Indianapolis, IN, 1975* p. 125 (Abstr. 183)
42. C J Barnett, G J Cullinan, K Gerzon, R C Hoying, W E Jones, W M Newlon, G A Poore, R L Robison, M J Sweeney, G C Todd *J. Med. Chem.* **21** 88 (1978)
43. V S Sethi, S S Burton, D V Jackson *Cancer Chemother. Pharmacol.* **4** 183 (1980)
44. R J Owellen, C A Hartke, F O Hains *Cancer Res.* **37** 2597 (1977)
45. P A Ponarotti, R Rahmani, M Martin, J Barbet *Mol. Immunol.* **22** 277 (1985)
46. M C Cibotti, C Freier, J Andrieux, M Plant, L Cosson, C Bonuon *Phytochemistry* **29** 2109 (1990)
47. R Rahmani, J Barbet, J P Cano *Clin. Chim. Acta* **129** 57 (1983)
48. A S Perlin, in *Oxidation. Techniques and Applications in Organic Synthesis* Vol. 1 (Ed. R L Augustine) (New York: Marcel Dekker, 1969) p. 189
49. M Lounasmaa, R Jokela, A Huhtikangas, S Lapinjoki, J Heiskanen, T Lehtola, T Naaranlahti *Heterocycles* **24** 1355 (1986)
50. S P Lapinjoki, H M Verajankorva, A Huhtikangas, T J Lehtola, M Lounasmaa *J. Immunoassay* **7** 113 (1986)
51. A Huhtikangas, T Lehtola, S Lapinjoki, M Lounasmaa *Planta Med.* **53** 85 (1987)
52. K Hirata, M Kobayashi, K Miyamoto, T Hoshi, M Okazaki, Y Miura *Planta Med.* **55** 262 (1989)
53. M Reihlin, J J Schnurre, V K Vance *Proc. Soc. Exp. Biol. Med.* **128** 347 (1968)
54. R J Owellen, M A Root, F O Hains *Cancer Res.* **37** 2603 (1977)
55. M Tabata, in *Plant Tissue Culture and Its Bio-Technological Application* (Eds W Barz, E Reinhard, M H Zenk) (Berlin: Springer, 1977) p. 3
56. W G W Kurz, K B Chatson, F Constabel, J P Kutney, L S L Choi, P Kolodziejczyk, S K Sleigh, K L Stuart, B R Worth *Phytochemistry* **19** 2583 (1980)
57. J P Kutney, L S L Choi, P Kolodziejczyk, S K Sleigh, K L Stuart, B R Worth, W G W Kurz, K B Chatson, F Constabel *Helv. Chim. Acta* **64** 1837 (1981)
58. P Manganey, R Z Andriamialisoa, N Langlois, Y Langlois, P Potier *J. Am. Chem. Soc.* **101** 2243 (1979)
59. A Huhtikangas, J Heiskanen, V Kauppinen, S Lapinjoki, M Lounasmaa, H Verajankorva *Pharm. Weekblad – Sci. Ed.* **9** 236 (1987)
60. S Lapinjoki, H Verajankorva, J Heiskanen, M Niskanen, A Huhtikangas, M Lounasmaa *Planta Med.* **53** 565 (1987)
61. B Deus-Neumann, J Stockigt, M H Zenk *Planta Med.* **53** 184 (1987)
62. M H Zenk, H El-Shagi, H Arens, J Stockigt, E W Weiler, B Deus, in *Plant Tissue Culture and Its Bio-Technological Application* (Eds W Barz, E Reinhard, M H Zenk) (Berlin: Springer, 1977) p. 27.
63. J F Treimer, M H Zenk *Phytochemistry* **17** 227 (1978)
64. H Arens, B Deus-Neumann, M H Zenk *Planta Med.* **53** 179 (1987)
65. M Cushman, N Castagnoli Jr *J. Org. Chem.* **37** 1268 (1972)
66. S Balabanova, E Schneider, G Buhler *Dtsch. Apoth. – Ztg.* **130** 2200 (1990)
67. B J Savary, D K Dougall *Phytochemistry* **29** 1567 (1990)
68. M A Fliniaux, A Jacquin-Dubreuil *Planta Med.* **53** 87 (1987)
69. V Rowell, F J Rowell *Analyst* **112** 1437 (1987)
70. A J Quattrone, R Joseph, T Jones, S Sarmir, A Selvaggi, K Maloney, T Lockwood *J. Med. Technol.* **1** 381 (1984)
71. B E Pape *Ther. Drug Monit.* **3** 357 (1981)
72. C R Lehmann, K J Boran, W P Pierson, A P Melikian, G W Wright *Ther. Drug Monit.* **8** 336 (1986)
73. M Sheehan, G Caron *Ther. Drug Monit.* **7** 108 (1985)
74. M B Bottorff, R L Lalonde, A B Straughn *Biopharm. Drug Dispos.* **8** 213 (1987)
75. H R Ha, S Vozeh, F Follath *Ther. Drug Monit.* **8** 331 (1986)
76. D J Mazak, J Y Lee, J A Ferringer *ASHP Midyear Clinical Meeting* **25** 26E (1990)
77. M Tod, G Resplandy, R Farinotti, Y Provost, A Dauphin *J. Pharm. Biomed. Anal.* **8** 279 (1990)
78. Y W Lam, C Watkins *ASHP Midyear Clinical Meeting* **26** 348E (1991)
79. A Yamasaki, K Yamamoto, K Shinozaki, R Takao, Y Tanaka *J. Pract. Pharm.* **42** 191 (1991).
80. J V Aranda, K Beharry, J Rex, R J Johannes, L Charest-Boule *Ther. Drug Monit.* **9** 97 (1987)
81. K H Carpenter, M J Bennett *Ann. Clin. Biochem.* **24** (Suppl. 1) 61 (1987)
82. N P Danilova, N I Bekman, L V Shustova, R G Vasilov *Khim. – Farm. Zh.* **23** 871 (1989)
83. N P Danilova, N I Bekman, A S Ziganshin, R G Vasilov *Khim. – Farm. Zh.* **26** 92 (1992)
84. J M Rattenbury, T Taylor *Ann. Clin. Biochem.* **25** (1988)
85. P A Kyd, S J Evans *Ann. Clin. Biochem.* **24** (Suppl. 1) 60 (1987)
86. N Massoud, P Munzenberger, A Sarniak *Am. J. Hosp. Pharm.* **43** 1722 (1986)
87. J Babjuk, I Bilyk, J Kalousova, H Nekvasilova, B Rybakova *Cesk. Farm.* **36** 322 (1987)
88. A Neimann, M Oellerich, G Schumann, G W Sybrecht *J. Clin. Chem. Clin. Biochem.* **23** 725 (1985)
89. P W Thies, B Nalge *Hosp. Pharm.* **21** 230 (1986)
90. R Kino, R O Day, G A Pearce, G W Fulde *Aust. J. Hosp. Pharm.* **21** 292 (1991)
91. M J Otero, A Martin Suarez, M Barrueco, E Gomez, A Dominguez-Gil *Farm. Clin.* **4** 528 (1987)
92. G Gotelli, M Mitchell *Clin. Chem.* **31** 1085 (1985)
93. P Goebbels, J Boulander, P Ers, A Adam *Clin. Chem.* **33** 1077 (1987)
94. M Plebani, A Burlina *Ther. Drug Monit.* **7** 451 (1985)
95. D L Morris *Anal. Biochem.* **151** 235 (1985)
96. T Kuzuya, Y Ogura, M Hasegawa, T A Nakamura, S Katazama *Jpn. J. Hosp. Pharm.* **12** 362 (1986)
97. R Chen, T M Li, H Merrick, R F Parrish, V Bruno, A Kwong, C Stiso, D J Litman *Clin. Chem.* **33** 1521 (1986)
98. R H Drost, H U Schulz, R A Maes, J M Van Rossum *J. Toxicol. Clin. Toxicol.* **25** 231 (1987)
99. E F Ullman, T Tarnowski, P Felgner, I Gibbons *Clin. Chem.* **33** 1579 (1987)
100. P J Aznar, B M Toreda, A M Alos, B J Ezqner, O M D Fuentez *Farm. Clin.* **8** 139 (1991)
101. R L Lalonge, M B Bottorff, A B Staughn *Ther. Drug Monit.* **7** 442 (1985)
102. J H Jonkman *Ther. Drug Monit.* **10** 345 (1988)
103. M Pokrajac, D Agbaba, V M Varagic, L Glisovic *Acta Pharm. Jugosl.* **36** 445 (1986)
104. E Pietruszewska *Farm. Pol.* **41** 382 (1985)
105. H M Izquiero, F O Calatayud, G G Sangrador, A A Sanchez *Farm. Clin.* **4** 802 (1987)
106. H Wallinder, L L Gustafsson, K Angback, P Hoglung, L Arvanius *Ther. Drug Monit.* **13** 233 (1991)
107. M Mordelet-Dambrine, J Y Baglin, A Roux, D Dusser, G Huchon *Ther. Drug Monit.* **8** 106 (1986)
108. M M Canto Pascual, M Alos Alminana, J Magraner Gil, J Ezquer Borras, M D Fuentes Ortiz, M L Calduch Alvarez, M T Torrecilla Junyent *Farm. Clin.* **4** 38 (1987)
109. P-L C Lopez, M T Miranda-Leon, M A Agudo-Martinez, D Villalba-Bedoya *Farm. Clin.* **7** 485 (1990)

110. R J West, L N Sansom, A C Penna *Aust. J. Hosp. Pharm.* **20** 430 (1990)
111. A W Krings *Ziekenhuisfarmacie* **7** 7 (1991)
112. I S Kampa, J I Jarzabek *Ther. Drug Monit.* **7** 489 (1985)
113. I A Cohen, C E Johnson, L Wesolowicz, K Converse-Swastek *Clin. Pharm.* **4** 457 (1988)
114. J S Wei, C Y Tsai, H M Huang, M E Chai *Ther. Drug Monit.* **11** 84 (1989)
115. M Morton, R C Parish, W J Spruill *Ther. Drug Monit.* **11** 347 (1989)
116. N D Danilova, S N Lutskova, I I Fadeeva, R G Vasilov, A I Miroshnikov *Khim.–Farm. Zh.* **24** 56 (1990)
117. T Tanahashi, A Poulev, M H Zenk *Planta Med.* **56** 77 (1990)
118. S Spector *J. Pharmacol. Exp. Ther.* **178** 253 (1971)
119. D H Catlin, R Cleeland, E Grunbarg *Clin. Chem.* **19** 216 (1973)
120. F L Adler, C T Lin, D H Catlin *Clin. Immunol. Immunopathol.* **1** 53 (1972)
121. K E Rubenstein, R S Schneider, E F Ullman *Biochem. Biophys. Res. Commun.* **47** 846 (1972)
122. R S Schneider, P Lindqwist, E Tong-in-Wong, K E Rubenstein, E F Ullman *Clin. Chem.* **19** 821 (1973)
123. G L Rowley, K E Rubenstein, S T Weber, E F Ullman, in *Am. Chem. Soc. Meeting BIOL* (Washington, 1976) p. 151
124. G L Rowley, K E Rubenstein, J Huisjen, E F Ullman *J. Biol. Chem.* **250** 3759 (1975)
125. J W Lee, J E Pedersen, T L Moravetz, A M Dzerk, A D Mundt, K V Shepard *J. Pharm. Sci.* **80** 284 (1991)
126. D L Black, B A Goldberger, Y H Caplan *Clin. Chem.* **33** 367 (1987)
127. C Lora-Tamayo, T Tena, G Tena *J. Chromatogr.* **422** 267 (1987)
128. U Wieczorek, N Nagakura, M H Zenk *Phytochemical Society Symposium, London, 1984* (Abstr. 36)
129. P A Glare *Ther. Drug Monit.* **13** 1 (1990)
130. D J Litman, R H Lee, H J Jeong, H K Tom, S N Stiso, N C Sizto, E F Ullman *Clin. Chem.* **29** 1598 (1983)
131. R Ross, C A Horwitz, H Hager, M Usategui, M D Burke, P C J Ward *Clin. Chem.* **21** 139 (1975)
132. D Laurie, A J Manson, A Mounsey, F J Rowell, J Seviour *Analyst* **112** 687 (1987)
133. D L Colbert, G Gallacher, P Ayling, G L Turner *Clin. Chim. Acta* **171** 37 (1988)
134. D L Colbert, D S Smith, A M Sidki *Ann. Clin. Biochem.* **23** 37 1986
135. M A Myagkova, M V Lushnikova *Khim.–Farm. Zh.* **24** 76 (1990)
136. A Franceshin, L Morosine *Clin. Chem.* **33** 303 (1987)
137. A V Sereda, I E Sukhov, B M Zolotarev, I V Yartseva, O N Tolkachev *Tetrahedron Lett.* **33** 4205 (1992)
138. S N Eremenko, I E Sukhov, N P Danilova, O N Tolkachev, R G Vasilov, A I Miroshnikov *Khim.–Farm. Zh.* **26** 100 (1992)
139. M J Kogan, D J Pierson, M M Durkin, N J Willson *J. Chromatogr. Biomed. Appl.* **490** 236 (1989)
140. C R Hamlin *Clin Chem.* **34** 158 (1988)
141. S K Rouan, I G Otterness, D J Gans, C T Rhodes *Drug Dev. Ind. Pharm.* **17** 2391 (1991)