Journal of Chromatography, 142 (1977) 611–619

CHROM. 10,333

DETERMINATION OF OXYPHENONIUM BROMIDE IN PLASMA AND URINE BY MEANS OF ION-PAIR EXTRACTION, DERIVATIZATION AND GAS CHROMATOGRAPHY–ELECTRON-CAPTURE DETECTION

JAN E. GREVING, JAN H. G. JONKMAN, FENNY FIKS and ROKUS A. DE ZEEUW

Laboratory for Pharmaceutical and Analytical Chemistry, State University, 9713 AW Groningen (The Netherlands)

and

L. E. VAN BORK and N. G. M. ORIE

Pulmonary Division of the Medical Department, State University, Groningen (The Netherlands)

SUMMARY

A sensitive and selective method for the determination of the quaternary ammonium compound oxyphenonium bromide (Antrenyl), a drug with strong anticholinergic properties, in human plasma and urine is described. The method is based on ion-pair extraction of the cation with perchlorate, a re-extraction according to ion-pair principles with tetrapentylammonium as the counter ion, hydrolysis to cyclohexylphenylglycolic acid, derivatization of this acid to its pentafluorobenzyl ester and determination of the ester by gas chromatography and electron-capture detection. Quantitation is possible down to 2 ng/ml of oxyphenonium bromide using 1 ml of plasma and down to 200 ng/ml using 0.1 ml of urine. The method described can also be applied to other anticholinergic drugs with an ester function.

INTRODUCTION

Oxyphenonium bromide, [2-(2-cyclohexyl-2-hydroxy-2-phenylacetoxy)ethyl]diethylmethylammonium bromide (OxBr, Antrenyl; Fig. 1) is a potent anticholinergic drug. Being a quaternary ammonium compound, the drug has strong hydrophilic properties and, as a result, extraction from biological fluids is difficult. Two colorimetric methods[1,2] for the determination of oxyphenonium (Ox^+) in biological

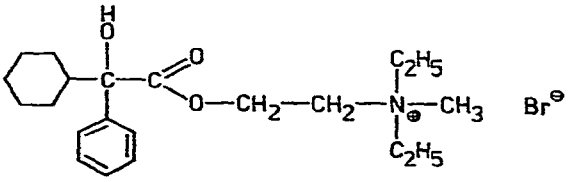

Fig. 1. Oxyphenonium bromide.

samples of animal origin have been published, based on ion-pair extraction with bromophenol blue. These methods are not selective or sensitive enough for determinations in human plasma. Owing to the high potency of OxBr, relatively low doses must be given so that plasma levels may be expected to be in the low nanogram range. Another prerequisite is that an assay in biological fluids should distinguish between the parent compound and possible metabolites, in particular α-cyclohexyl-α-phenylglycolic acid (CHPGA). The latter compound is inactive, but may result from metabolic cleavage of the ester function due to cholinesterase activity.

This paper presents a highly sensitive and selective method for the determination of Ox^+ in human plasma and urine, based on ion-pair extraction, hydrolysis of the ester, derivatization of the acid moiety CHPGA to its pentafluorobenzyl ester (CHPGA-PFB) and gas chromatography–electron-capture detection (GC–ECD). Potentially interfering compounds of an endogenous nature such as fatty acids and hippuric acid are removed by a selective extraction procedure before derivatization.

EXPERIMENTAL

Apparatus

A Hewlett-Packard Model 5830 gas chromatograph equipped with a 15-mCi ^{63}Ni pulse-frequency modulated electron-capture detector was used. This instrument was operated with a glass column (180 cm × 0.2 cm I.D.), coated with HMDS and packed with 3% OV-17 on Chromosorb W-HP (80–100 mesh). The carrier gas was argon–methane (95:5), dried over molecular sieve 3 Å, at a flow-rate of 30 ml/min. The temperatures were: 300° for the injector, 235° for the column and 300° for the detector.

Glassware

Derivatization tubes (1-ml Reacti Vials, Pierce, Rockford, Ill., U.S.A.) and screw-capped centrifuge tubes (Sovirel 13, Quickfit S.A., Epernon, France) were cleaned in a mixture of 80 ml of hydrogen peroxide (36%), 300 ml of hydrochloric acid (36%) and 120 ml of distilled water by standing overnight, then rinsed with distilled water and dried at 105°. Centrifuge tubes (Sovirel 15) and the PTFE-lined screw-caps were cleaned with a 2% solution of Extran MA 01 (Merck, Darmstadt, G.F.R.) by heating to 95°, cooling and standing overnight, then rinsed with distilled water and dried at 105°. Contact of wet glassware with the skin must be avoided.

Chemicals and reagents

Oxyphenonium bromide (OxBr) and α-cyclohexyl-α-phenylglycolic acid (CHPGA) were kindly supplied by Ciba-Geigy (Basle, Switzerland). Benactyzine hydrochloride and pentafluorobenzyl bromide (PFB-Br) were obtained from Aldrich-Europe (Beerse, Belgium). Benzilic acid (BA) and all other chemicals and solvents were of reagent grade and obtained from Merck. Tetrapentylammonium iodide (TPeA-I) was obtained from Eastman-Kodak (Rochester, N.Y., U.S.A.).

Benactyzine methoiodide (BenMI, [2-(2,2-diphenyl-2-hydroxyacetoxy)ethyl]-diethylmethylammonium iodide) was used as an internal standard and was prepared by methylation of benactyzine in benzene with methyl iodide. After stirring for 3 h at room temperature, the crystalline product was filtered off and recrystallized from

ethanol (99.5%). After drying, the product had a melting point of 130–131.5°. Elemental analysis and IR, NMR and MS confirmed its structure.

Dichloromethane (DCM) was purified over a column with molecular sieve 3 Å followed by a column with silica gel 60 (extra-pure for column chromatography) (70–230 mesh) and finally by distillation in glass. To the distillate (40°), 0.2% (v/v) of ethanol (99.5%) was added. Dichloroethane (DCE) was used after distillation in glass.

Tetrapentylammonium ($TPeA^+$) solution ($2 \cdot 10^{-3}$ *M*; pH 7.5) was prepared by adding 2.13 g of TPeA-I and 0.637 g silver oxide to 30 ml of distilled water, with reaction for 1 h in an ultrasonic water-bath at room temperature. The precipitate was filtered off with a glass filter and the filtrate collected in a separating funnel and extracted twice with equal volumes of DCM. The aqueous layer was neutralized with orthophosphoric acid (0.1 *M*) to pH 7.5 and again extracted twice with equal volumes of DCM. The $TPeA^+$ concentration in the remaining aqueous phase was determined by the method of Gustavii and Schill[3] and the solution was diluted to give a final $TPeA^+$ concentration of $2 \cdot 10^{-3}$ *M*.

Pentafluorobenzyl bromide solution (0.1%, v/v) was prepared by adding 10 µl of PFB-Br to 10 ml of purified DCM.

Reference samples of CHPGA-PFB and BA-PFB were prepared as described previously[4].

Determination of oxyphenonium in plasma

Blood samples were obtained by venous puncture and collected in heparinized glass centrifuge tubes (Sovirel 15). After centrifugation, 1.0 or 2.0 ml of plasma was transferred into a second tube, 50 µl of an aqueous solution containing 50 ng of Ben-MI (internal standard), 100 µl of 1 *N* sodium perchlorate solution and 4 ml of DCE were added and the mixture was shaken for 30 sec on a Vortex-Genie mixer. The phases were separated by centrifugation for 10 min at 5000 *g*, and the aqueous layer was aspirated by means of a Pasteur pipette. A 3-ml volume of the DCE phase was transferred into another centrifuge tube and 1 ml of the $TPeA^+$ solution was added. After mixing for 30 sec on the Vortex and centrifugation for 5 min at 5000 *g*, 800 µl of the aqueous phase were transferred into a centrifuge tube (Sovirel 13) to which 1 ml of purified DCM was added. After extraction under the same conditions as described above, 600 µl of the aqueous phase was transferred into another tube to which 50 µl of 1 *N* sodium hydroxide solution was added. The mixture was hydrolysed for 30 min at 40° in a thermostated water-bath. After cooling to room temperature, 100 µl of 1 *M* sodium dihydrogen orthophosphate and 1 ml of purified DCM were added. Extraction was carried out as above, followed by cooling for 5 min in an ice-bath. Then the tube was centrifuged for 5 min at 5000 *g* in a cooled centrifuge at about 0°. The aqueous phase was aspirated and 500 µl of the DCM phase were transferred into a derivatization tube and evaporated to dryness under nitrogen. To the residue 50 µl of PFB-Br solution were added and the tube was closed well and heated for 3 min at 65° in a block heater. After cooling to room temperature, the solution was evaporated to dryness under nitrogen and the residue was dissolved in 50 µl of *n*-heptane–ethyl acetate (98:2).

A 2.5-µl volume was injected into the gas chromatograph, and quantitation was effected by electronic integration. A standard response graph was prepared by adding known amounts of OxBr to 1.0 ml of drug-free plasma.

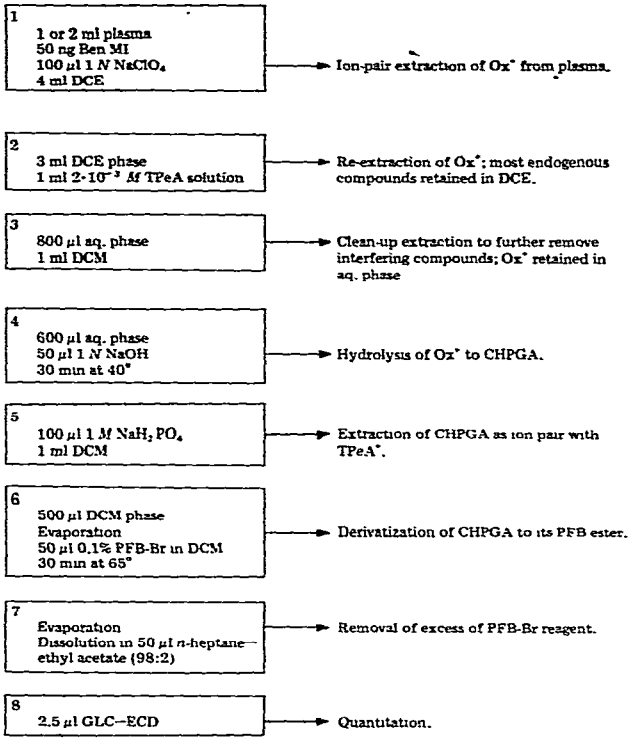

Fig. 2. Scheme for the determination of oxyphenonium bromide in plasma.

Determination of oxyphenonium in urine

A 100-µl volume of each urine fraction was taken in a centrifuge tube (Sovirel 15), 800 µl of phosphate buffer (0.1 *M*, pH 7.0), 50 µl of an aqueous solution containing 100 ng of BenMI, 100 µl of 1 *N* sodium perchlorate solution and 4 ml of DCE were added and the mixture was shaken for 30 sec on a Vortex-Genie mixer. The phases were separated by centrifugation for 10 min at 5000 *g*, and the aqueous layer was aspirated by means of a Pasteur pipette. A 3-ml volume of the DCE phase was transferred into another tube and extracted with 1 ml of 0.1 *M* phosphate buffer (pH 7.0) containing 10^{-3} *M* sodium perchlorate. After separation of the phases, 2 ml of the DCE phase was transferred into a centrifuge tube (Sovirel 15) and 1 ml of the $TPeA^+$ solution was added. After extraction for 30 sec on a Vortex-Genie mixer and centrifugation for 5 min at 5000 *g*, 800 µl of the aqueous phase were transferred into a centrifuge tube (Sovirel 13) to which 50 µl of 1 *N* sodium hydroxide solution was added. Hydrolysis to CHPGA, extraction of the acid with $TPeA^+$, derivatization to its PFB ester and removal of excess of PFB-Br were carried out as described for plasma (see steps 4–7 in Scheme 1). After derivatization and evaporation, the residue was dissolved in 100 µl of n-heptane-ethyl acetate (98:2) and 2.5 µl were injected into the gas chromatograph. A standard response graph was prepared by adding known amounts of OxBr to 0.1 ml of drug-free urine.

RESULTS AND DISCUSSION

The general procedure for the determination of OxBr in plasma is shown schematically in Fig. 2.

Ion-pair extraction of oxyphenonium

For the isolation of quaternary ammonium compounds such as Ox^+ from aqueous solutions, ion-pair extraction with a suitable counter ion can be used. For Ox^+ and $BenM^+$, three counter ions, namely iodide, perchlorate and picrate, were tested in extractions with DCE as the organic solvent essentially according to Gustavii and Schill[3,4]. Dissociation of the ion pairs in the organic phase was the only side-reaction found to occur. Extraction constants (E_{QX}) and dissociation constants (k_{diss}) are given in Table I. With iodide the E_{QX} value is too low for quantitative extraction. Picrate gives a very high E_{QX} and therefore may result in a less selective extraction from biological fluids. Picrate may also interfere later in the derivatization procedure with PFB-Br. Hence perchlorate seems to be the best choice as the counter ion. It should be noted that dissociation in the organic phase will increase the recovery with decreasing concentration of Ox^+ (ref. 5). This is an advantage, because the therapeutic plasma levels are very low.

TABLE I

EXTRACTION CONSTANTS AND DISSOCIATION CONSTANTS OF OXYPHENONIUM BROMIDE AND BENACTYZINE METHOIODIDE

Counter ion	*Oxyphenonium bromide*		*Benactyzine methoiodide*	
	E_{QX}	k_{diss}	E_{QX}	k_{diss}
Iodide	80	—*	4	$1 \cdot 10^{-5}$
Picrate	$1 \cdot 10^5$	$1 \cdot 10^{-5}$	$1 \cdot 10^4$	$4 \cdot 10^{-5}$
Perchlorate	$7.5 \cdot 10^2$	$3.2 \cdot 10^{-5}$	$1 \cdot 10^2$	$2.3 \cdot 10^{-5}$

* Accurate determination was not possible.

As a result of extraction with DCE a large amount of interfering lipophilic acids are co-extracted[6] from plasma and a re-extraction was found to be necessary. However, the dissociation of Ox perchlorate in DCE prevented quantitative re-extraction with water alone. We therefore applied a displacement procedure by adding $TPeA^+$ (as phosphate) to the water. In combination with perchlorate, $TPeA^+$ has an extraction constant, E_{QX}, that is several times higher than that of Ox^+ with perchlorate[3], which results in an exchange of Ox^+ to the aqueous phase and $TPeA^+$ as ion pair with perchlorate to the DCE phase.

Endogenous lipophilic fatty acids are retained in the DCE phase as protonated acids and/or as ion pairs with $TPeA^+$. For plasma, additional amounts of fatty acids, in particular palmitic acid, the PFB derivative of which will interfere in the quantitation, can be removed in step 3 in Fig. 2. The above clean-up steps effectively reduce interference by palmitic acid (the average content of free palmitic acid in plasma is about 100 μg/ml) to 2–5 ng/ml in the final sample.

In urine, much smaller amounts of fatty acids are present and step 3 of the determination in plasma can therefore be omitted. On the other hand, hippuric acid, the PFB derivative of which has a shorter retention time than that of CHPGA-PFB, and which is normally present in a high concentration (*ca.* 1 mg/ml) must be removed by a selective extraction step. Hippuric acid is a hydrophilic compound and its partition ratio in the first extraction step is low. Further clean-up was carried out by extraction of the DCE phase with a buffer of pH 7 and with a low concentration of perchlorate, so that Ox^+ is retained in the organic phase and trace amounts of hippuric acid in the organic phase migrate to the aqueous layer. Free CHPGA, which may be present as an inactive metabolite, does not interfere in the plasma and urine determinations. The clean-up steps to remove fatty acids and/or hippuric acid also remove CHPGA. No CHPGA-PFB was found after addition of CHPGA to drug-free plasma or urine.

Hydrolysis of oxyphenonium

In order to be able to prepare the PFB derivative of CHPGA, the acid moiety of Ox^+, the rate of hydrolysis of the latter was examined. The rate of hydrolysis of OxBr over the pH range 2.35–7.35 was determined by Grabowska[7]. Kuznetsov and Roginskaya[8] found that the rate of hydrolysis of quaternary ammonium esters of diphenylacetic and diphenylglycolic acids are much higher in alkaline than in acidic media. For tertiary analogues this difference is less pronounced.

We determined the hydrolysis rate constants over the pH range 7.5–11.85 at different temperatures in 0.05 *M* phosphate buffer. Quantitation was effected by ion-pair extraction of the non-hydrolysed part of Ox^+ with picrate as counter ion at pH 7.5, DCM as extraction solvent and spectrophotometric measurement of the yellow colour in the organic phase according to Gustavii and Schill[3].

For Ox^+ at pH 11.85 the hydrolysis rate constant, k, was 0.044/min at 20° and 0.019/min at 10°. Extrapolation gives a k value of 0.24/min at 40°. After 30 min at this temperature, 99.9% of Ox^+ will be hydrolysed at this pH.

For BenMI, which is used as an internal standard, Kuznetsov and Roginskaya[8] found a k value of 0.033/min at pH 11.5 and 30°. After extrapolation with the constants obtained for Ox^+, the k value for $BenM^+$ can be estimated to be 0.17/min at 40° and pH 11.85, which results in a hydrolysis of 99.4% of Ben M^+ after 30 min.

Derivatization of CHPGA to its PFB ester

PFB esters of carboxylic acids are known to exhibit high sensitivities in ECD. However, there are problems with derivatization and quantitation of picomole amounts: (*i*) long reaction times and non-quantitative yields; (*ii*) interference of excess of PFB-Br, which cannot be handled by ECD; and (*iii*) contamination of samples, glassware and reagents by acidic compounds of a biological nature such as fatty acids.

We applied a single ion-pair extraction of CHPGA with $TPeA^+$ as the counter ion into DCM. After evaporation, derivatization was carried out in DCM with a low concentration of PFB-Br by heating at 65° for 30 min. The sample was then evaporated to dryness.

The advantages of this method are as follows: (*i*) the single ion-pair extraction

permits a selective isolation of CHPGA in the non-protonated form; (*ii*) CHPGA as an ion pair with $TPeA^+$ shows a high reactivity for alkylation even with a low concentration PFB-Br in DCM; and (*iii*) excess of the low-concentration PFB-Br can easily be removed by evaporation. The present method of derivatization[9] offers a much higher sensitivity than other proposed methods for esterification of PFB[10–16].

In order to prevent contamination of glassware, special cleaning procedures are necessary, as described under Experimental. Moreover, it was found that various chemicals and reagents contained interfering amounts of fatty acid impurities, which had to be removed as indicated.

Results of the overall procedures

Fig. 3 shows the gas chromatograms obtained in the analysis of drug-free plasma (A) and drug-free plasma to which 50 ng of OxBr and 50 ng of BenMI had been added (B). It can be seen that there is little or no interference from palmitic acid. Fig. 4 gives the standard response graph on a logarithmic scale for single determinations in 1.0 ml of plasma over an OxBr concentration range of 2–100 ng. Power curve fitting of the equation $y = Ax^b$ gave a correlation coefficient of 0.998, with $b = 1.004$. The coefficient of variation was 6.9% ($n = 8$) at the 10 ng/ml level. Recoveries of CHPGA-PFB from Ox^+ were about 75–85% at the same level; 10% of the loss can be explained by the last extraction step[9].

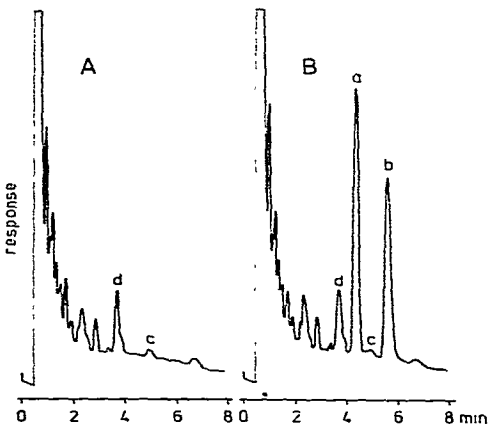

Fig. 3. Gas chromatograms of oxyphenonium in plasma. A, Drug-free plasma. B, Plasma spiked with 50 ng/ml of oxyphenonium bromide and 50 ng/ml of benactyzine methoiodide (internal standard). (a) CHPGA-PFB ester; (b) BA-PFB ester; (c) palmitic acid PFB ester (contaminant); (d) contaminant from cap inlay derivatization tube.

For urine, a standard response graph was prepared over the concentration range 0.2–10 μg/ml using 0.1 ml of urine. The correlation coefficient was 0.999. The coefficient of variation was 7.4% ($n = 8$) at a level of 1.0 μg/ml of OxBr. Recoveries of CHPGA-PFB were about 80%.

The described methods were applied to follow OxBr levels in plasma and urine after intramuscular doses of 2 mg to asthmatic patients. Maximum plasma

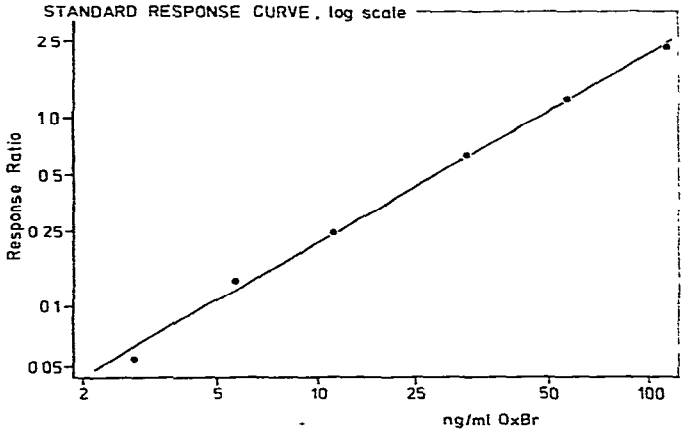

Fig. 4. Standard response graph (logarithmic scale) for oxyphenonium bromide determinations in plasma using 50 ng/ml of benactyzine methoiodide as internal standard.

levels were 50–100 ng/ml after about 5–15 min and decreased to about 2 ng/ml after 7 h. Urine levels ranged from about 10 μg/ml during the first hour, decreasing to 0.2 μg/ml after 24 h. Fig. 5 shows a plasma concentration–time graph on a semi-logarithmic scale for an asthmatic patient who had received 2 mg of OxBr by intramuscular injection.

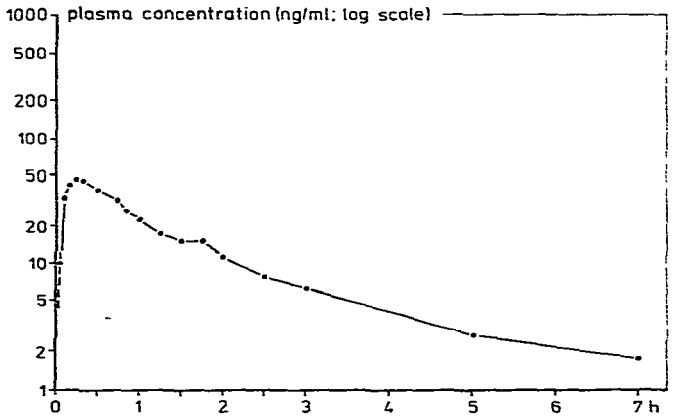

Fig. 5. Plasma concentration–time graph (semi-logarithmic scale) after intramuscular administration of 2 mg of oxyphenonium bromide to an asthmatic patient.

It should be noted that interference may be expected from other drugs that containing the CHPGA moiety, such as oxyphencyclimine, whereas the BA moiety is found in benactyzine, benzilonium bromide, bevonium bromide, clidinium bromide, 2-dimethylaminoethyl benzilate, pipenzolate bromide, piperilate, piribenzil methosulphate, poldinium methosulphate and tropenzile.

ACKNOWLEDGEMENTS

We thank Ciba-Geigy, Basle, Switzerland, for reference samples of oxyphenonium bromide and cyclohexylphenylglycolic acid.

This work was supported in part by Grant 243 of the Netherlands Asthma Foundation (NAF).

REFERENCES

1 R. M. Levine and B. B. Clark, *J. Pharmacol. Exp. Ther.*, 121 (1957) 63.
2 H. Wagle, H. Sheppard and A. Plummer, *Fed. Proc., Fed. Amer. Soc. Exp. Biol.*, 13 (1954) 413.
3 K. Gustavii and G. Schill, *Acta Pharm. Suecica*, 3 (1966) 241.
4 K. Gustavii and G. Schill, *Acta Pharm. Suecica*, 3 (1966) 259.
5 K. O. Borg, *Acta Pharm. Suecica*, 6 (1969) 425.
6 D. Westerlund and A. Söderqvist, *Acta Pharm. Suecica*, 12 (1975) 277.
7 I. Grabowska, *Acta Pol. Pharm.*, 28 (1971) 305.
8 S. G. Kuznetsov and E. V. Roginskaya, *Zh. Obshch. Khim.*, 32 (1962) 2026.
9 J. E. Greving, J. H. G. Jonkman and R. A. de Zeeuw, *J. Chromatogr.*, in press.
10 F. K. Kawahara, *Anal. Chem.*, 40 (1968) 2073.
11 J. A. F. Wickramasinghe, W. Morozowich, W. E. Hamlin and S. R. Shaw, *J. Pharm. Sci.*, 62 (1973) 1428.
12 L. G. Johnson, *J. Ass. Offic. Anal. Chem.*, 56 (1973) 1503.
13 D. G. Kaiser, S. R. Shaw and G. J. VanGiessen, *J. Pharm. Sci.*, 63 (1974) 567.
14 A. S. Y. Chau and K. Terry, *J. Ass. Offic. Anal. Chem.*, 59 (1976) 633.
15 O. Gyllenhaal, H. Brötell and P. Hartvig, *J. Chromatogr.*, 129 (1976) 295.
16 B. Davis, *Anal. Chem.*, 49 (1977) 832.