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LIQUID CHROMATOGRAPHIC SCREENING OF DIURETICS IN URINE

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SUMMARY

We describe a liquid chromatographic screening procedure for the detection, in urine, of twelve of the fifteen potassium-depleting diuretics available in Australia. A 2-ml urine sample was acidified with NaH_2PO_4 (pH 4.1) and extracted with 4 ml ethyl acetate. The sample was cleaned up further by washing with 5 ml Na_2HPO_4 (pH 7.5). The ethyl acetate was then evaporated to dryness, the residue reconstituted in 100 μl mobile phase and 5 μl were injected onto a Merck LiChrosorb RP-18 (5 μm) column. The ultraviolet absorbance of the eluent was monitored at 271 nm for 10 min. The screen was evaluated by giving each of thirty volunteers the lowest recommended dose of one of the diuretics in the study and obtaining urine samples 4, 8 and 24 h after having taken the dose. Twelve diuretics, chlorothiazide, hydrochlorothiazide, quinethazone, chlorthalidone, methyclothiazide, clopamide, frusemide, metolazone, mefruside, bendrofluazide, cyclopenthiazide and bumetanide, were all detectable up to 24 h after a dose. We therefore conclude that the screen would be reliable for the detection of these diuretics in urine.

INTRODUCTION

Diuretics are widely used in the treatment of congestive heart failure and hypertension. Most increase urinary potassium excretion and can cause hypokalemia in patients taking them for a prolonged period, either covertly or on prescription. Thus, when reliable information is not available from the patient or patient's history, the investigation of electrolyte imbalances may be helped by screening the urine for the presence of diuretics.

There are several methods available for the detection and quantitation of individual diuretics [1], however, only a few screening procedures exist [2–7]. Three of these only screen mixtures of pure compounds in pharmaceutial preparations [2–4]. The remaining three procedures detect diuretics in urine to varying degrees using quite independent methodologies. The method of Pilsbury and Jackson [5] detects the presence of thiazide diuretics using a spectrophotometric–paper chro-

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matographic procedure. This method is limited to thiazide diuretics and lacks the sensitivity and specificity of high-performance liquid chromatography (HPLC). Sohn et al. [6] use a thin-layer chromatographic procedure preceded by extraction on a non-ionic resin column, in the detection of a limited range of commonly used diuretics. Tisdall et al. [7] describe an HPLC screening procedure for the detection of thiazide diuretics in urine. The main drawbacks of their procedure are the use of two mobile phases in separate 12-min runs, the inability to detect non-thiazide diuretics and the inability to detect chlorothiazide directly.

This paper describes an HPLC procedure for identifying, in urine, twelve of the fifteen potassium-depleting diuretics available in Australia.

EXPERIMENTAL

Reagents

All reagents were of analytical or HPLC grade. Water was deionized and filtered (18 MΩ purity). Diuretic standard solutions were prepared from pure compounds kindly provided by the manufacturers: chlorothiazide, hydrochlorothiazide, chlorthalidone, frusemide, methyclothiazide and bendrofluazide, Protea Pharmaceuticals (Glebe, Australia); clopamide, Sandoz (North Ryde, Australia); cyclopenthiazide, Ciba-Geigy (Lane Cove, Australia); mefruside, Bayer (Botany, Australia); quinethazone, Cyanamid (Baulkham Hills, Australia); bumetanide, Astra Pharmaceuticals (North Ryde, Australia); metolazone, Searle (Crows Nest, Australia); clorexolone, May & Baker (West Footscray, Australia); indapamide, Servier (Hawthorn, Australia); and ethacrynic acid, Merck Sharp & Dohme (South Granville, Australia). Stock solutions in ethanol containing 1 mg/ml of each drug were stored at 4°C.

Apparatus

A Hewlett-Packard 1090A liquid chromatograph equipped with a diode array detector was used for all screens. A Merck LiChrosorb RP-18 (5 μm) column, 125 mm × 4 mm I.D. was used at 50°C to effect separation of the twelve diuretics studied. The flow-rate of the mobile phase, acetonitrile in 0.01 mol/l phosphate buffer (pH 3.0), was maintained at 1.5 ml/min while a gradient was used to increase the acetonitrile content from 10% at 1.5 min to 35% at 3.5 min. The diode array detector was set to monitor the signal to the integrator at 271 nm, however, spectral data between 210 and 400 nm were stored in computer memory (Hewlett-Packard 85B) and this could be plotted at the end of the run.

Urine extraction

Urine samples of 2 ml were acidified with 2 ml of 1 mol/l NaH_2PO_4 (pH 4.1) and 4 ml ethyl acetate were added. Tubes were vortexed for 2 min and centrifuged at 1500 *g* for 5 min. The organic phase was transferred to a second tube containing 5 ml of 0.1 mol/l Na_2HPO_4 (pH 7.5). The tubes were then vortexed for 2 min and centrifuged as above. The organic phase was transferred to a conical tube and evaporated to dryness at 60°C under a gentle stream of nitrogen. The residue was

then reconstituted with 100 μl of 35% acetonitrile in 0.01 mol/l phosphate buffer (pH 3.0) of which 5 μl were injected.

Recovery study

A urine sample was spiked to contain 10 μg/ml of each of the drugs and extracted as described. The peak heights obtained were then expressed as a percentage of the respective peak heights of an aqueous solution containing 200 μg/ml of each drug injected directly into the chromatograph.

Patient samples

Urine samples were obtained from 41 patients attending the Hypertension Clinic at the hospital. Each patient was receiving therapy with at least one of the diuretics under consideration as well as a range of other antihypertensive medications. The patient samples were chromatographed to verify the screening procedure as well as to ensure that there was no interference from concurrent medication.

Volunteer samples

Working in pairs, thirty volunteers were each given the lowest recommended dose of one of the fifteen diuretics. Spot urine samples were collected at 4, 8 and 24 h post-dose and put through the screening procedure. The diuretic concentration for each urine sample was determined where possible.

Sensitivity study

The 1 mg/ml stock standards of each drug in ethanol were used to prepare a composite solution containing 150 μg/ml chlorothiazide, mefruside and bumetanide; 100 μg/ml chlorthalidone, frusemide and metolazone; and 50 μg/ml of the others. The only standard not incorporated into the composite solution was clopamide as the sensitivity of this drug was estimated separately. The composite solution was used to spike urine samples at varying concentrations to estimate sensitivity and quantities present in the patient and volunteer specimens.

RESULTS

Chromatography

Fig. 1 shows a trace of the extract of a spiked urine sample. The urine sample contained the following concentrations; 30 μg/ml chlorothiazide, mefruside and bumetanide; 20 μg/ml chlorthalidone, frusemide and metolazone; and 10 μg/ml hydrochlorothiazide, quinethazone, methyclothiazide, bendrofluazide and cyclopenthiazide.

Fig. 2 shows a trace of an extract of drug-free urine and Fig. 3 of an extract of a urine sample containing 20 μg/ml clopamide. Clopamide coelutes with methyclothiazide in this system with a retention time of 4.7 min. We resolved this situation as follows. When a peak eluted at 4.7 min in an unknown specimen, the ultraviolet spectrum of the peak, between 220 and 400 nm, was plotted after the run using a Hewlett-Packard 7470A X–Y plotter. The spectrum was then matched

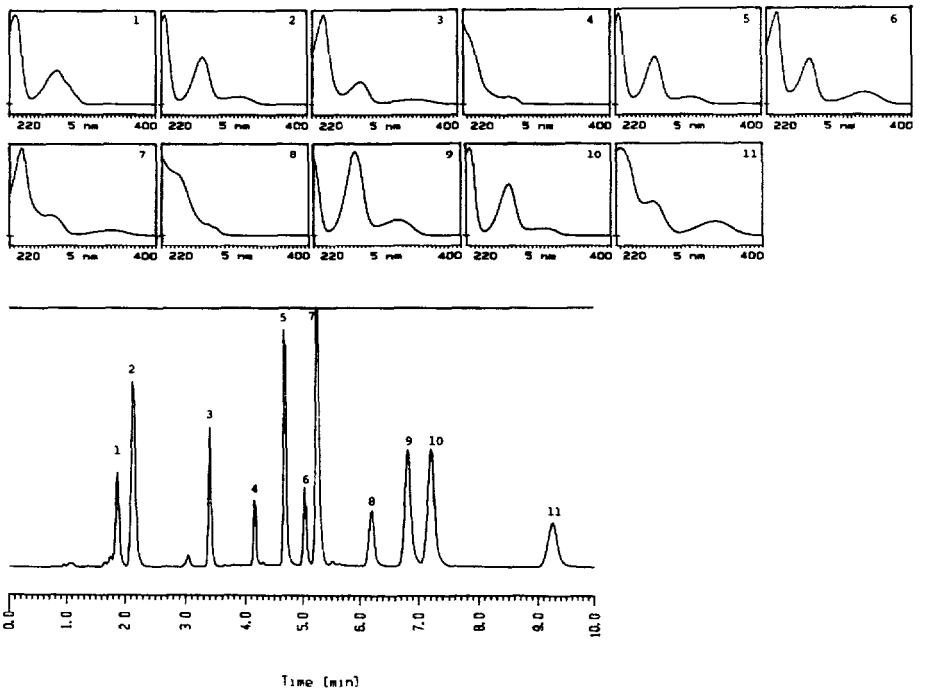

Fig. 1. Urine extract of eleven diuretics at listed concentrations run at 0.30 a.u.f.s. One twentieth of these amounts can be detected when run at 0.032 a.u.f.s. Pure solutions of indapamide, clorexolone and ethacrynic acid elute at 5.8, 60 and 8.3 min, respectively, but these are not detectable in clinical specimens. Peaks: 1 = chlorothiazide (30 μg/ml); 2 = hydrochlorothiazide (10 μg/ml); 3 = quinethazone (10 μg/ml); 4 = chlorthalidone (20 μg/ml); 5 = methyclothiazide (10 μg/ml); 6 = frusemide (20 μg/ml); 7 = metolazone (20 μg/ml); 8 = mefruside (30 μg/ml); 9 = bendrofluazide (10 μg/ml); 10 = cyclopenthiazide (10 μg/ml); 11 = bumetanide (30 μg/ml).

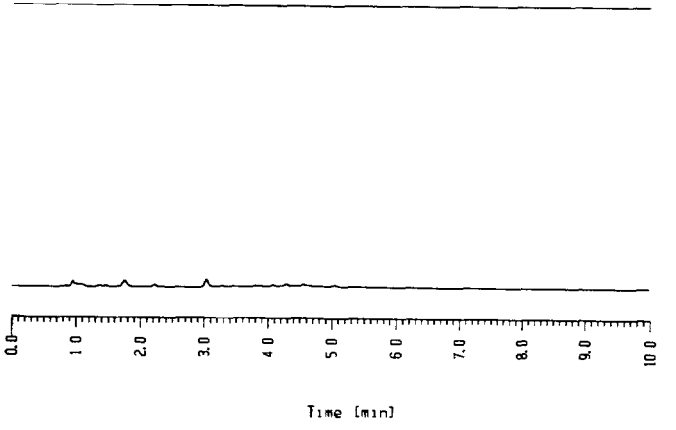

Fig. 2. Blank urine extract.

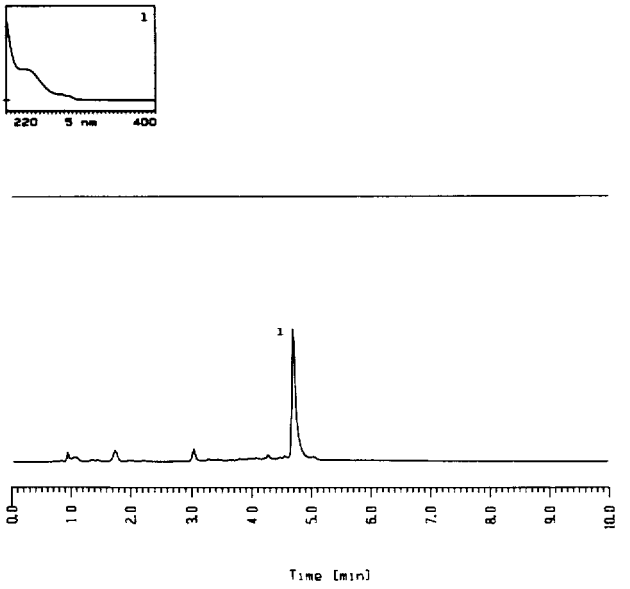

Fig. 3. Urine extract of 20 μg/ml clopamide (peak 1) run at 0.20 a.u.f.s. One twentieth of this amount can be detected when run at 0.032 a.u.f.s.

to known standard spectra of clopamide and methyclothiazide (Fig. 4) to determine which peak was in fact present. For laboratories without this facility it is satisfactory to report that a diuretic, either clopamide or methyclothiazide, was present in the urine sample. For quantitative estimation we used methyclothiazide in the composite standard, however, if the patient peak was identified as clopamide, an independent clopamide standard was run.

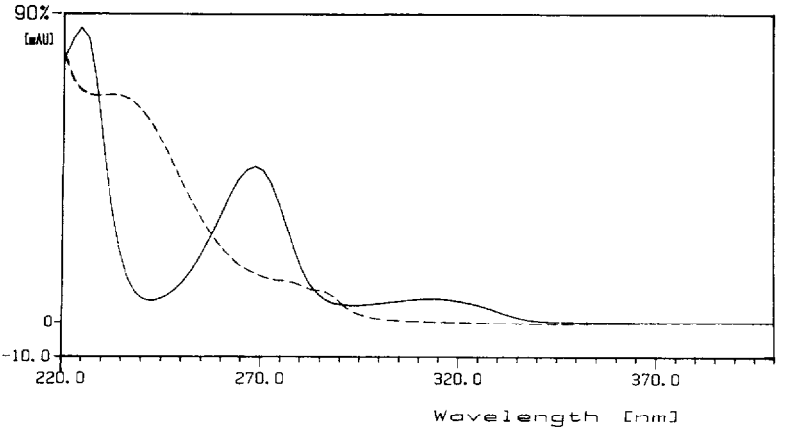

Fig. 4. Superimposed ultraviolet spectra of methyclothiazide (—) and clopamide (- - -) showing that they are easily distinguished. A post-run spectrum of a peak eluting at 4.70 min can therefore be identified.

TABLE I

EXTRACTION RECOVERY AND SENSITIVITY OF SCREEN ($n=5$)

Drug	Concentration (μg/ml)	Recovery (mean ± S.D.) (%)	Concentration (μg/ml)	Percentage full scale at 0.032 a.u.f.s. (mean ± S.D.)
Chlorothiazide	10	19 ± 0.6	1.5	15 ± 0.8
Hydrochlorothiazide	10	69 ± 0.6	0.5	28 ± 0.8
Quinethazone	10	52 ± 0.6	0.5	18 ± 0.9
Chlorthalidone	10	82 ± 2.3	1.0	13 ± 0.9
Methyclothiazide	10	87 ± 1.5	0.5	43 ± 1.1
Clopamide	10	91 ± 18.0	1.0	11 ± 0.5
Frusemide	10	12 ± 0.5	1.0	17 ± 1.1
Metolazone	10	80 ± 1.0	1.0	45 ± 1.1
Mefruside	10	89 ± 7.8	1.5	9 ± 0.0
Bendrofluazide	10	82 ± 2.3	0.5	17 ± 1.0
Cyclopenthiazide	10	80 ± 2.9	0.5	18 ± 1.9
Bumetanide	10	32 ± 1.2	1.5	9 ± 0.5

Recovery study

Recovery studies were based on extraction of 2-ml urine samples containing 10 μg/ml of each drug. The percentages of each drug recovered in the extraction are listed in Table I. The low percentages of chlorothiazide and frusemide recovered are due to loss of these relatively more acidic drugs, in the Na_2HPO_4 (pH 7.5)

TABLE II

POSITIVE SCREENS AND CONCENTRATION RANGE OF PATIENT SAMPLES

Diuretic	Number of patients	Number of positive screens	Concentration (μg/ml)	
			Range	Mean
Chlorothiazide	20	19	10 -1031	188
Frusemide	10	10	4 -54	22
Hydrochlorothiazide	8	7	3 -214	85
Cyclopenthiazide	2	2	0.08–0.09	0.09
Chlorthalidone	1	1	4	–

TABLE III

PATIENTS-PRESCRIBED MEDICATIONS WHICH SHOWED NO INTERFERENCE IN THE SCREENING PROCEDURE

Acetylsalicylic acid	Danthron	Metformin	Oxprenolol
Allopurinol	Diazepam	Methyldopa	Pindolol
Alprenolol	Digoxin	Metoprolol	Prazosin
Atenolol	Doxepin	Mianserin	Propranolol
Captopril	Glibenclamide	Minoxidil	Salbutamol
Carbimazole	Hydralazine	Nifedipine	Senokot
Clonidine	Indomethacin	Nitrazepam	Theophylline
Coloxyl	Labetalol	Oxazepam	Trifluoperazine

TABLE IV

DOSES ADMINISTERED AND LEVELS DETECTED IN DIURETIC SCREEN VOLUNTEERS

Drug	Administered dose (mg)	Mean level in urine after single dose (μg/ml)		
		4 h	8 h	24 h
Chlorothiazide	500	89.0	118.3	26.4
Hydrochlorothiazide	50	23.4	26.8	8.0
Quinethazone	50	21.0	25.5	7.5
Chlorthalidone	25	1.1	0.8	1.7
Methyclothiazide	5	0.1	0.2	0.2
Clopamide	20	0.2	0.2	0.1
Frusemide	40	8.3	5.6	0.4
Metolazone	2.5	0.8	1.3	0.3
Mefruside	25	0.3	0.2	Trace
Bendrofluazide	5	1.1	0.7	0.2
Cyclopenthiazide	0.5	Trace	0.03	0.4
Bumetanide	1	1.3	0.7	Trace

wash step of the extraction. This step is necessary to remove all strongly acidic substances so a clear chromatogram may result. The low recoveries of these drugs did not prove to be a problem in their detection as will be seen in the patient and volunteer samples studied (vide infra).

Patient samples

Of the 41 patients samples obtained from the Hypertension Clinic, twenty patients were taking chlorothiazide, eight hydrochlorothiazide, ten frusemide, two cyclopenthiazide and one chlorthalidone.

The number of positive findings detected using the screening procedure together with the range and mean concentrations estimated are shown in Table II.

The degree of patient compliance was not formally assessed at the time of sample collection but the screening results indicate high compliance. In only two patients, one presumed to be taking chlorothiazide and the other to be taking hydrochlorothiazide were there negative findings. Cyclopenthiazide was undetectable under the general conditions used but by increasing injection volume and recording the screen at a higher sensitivity setting, cyclopenthiazide could be detected.

The patients were taking their usual medications (Table III) at the time of sample collection. It should be noted that none of these other drugs interfered in the screening procedure. The only extraneous peak occurred at a retention time of 3.03 min. This peak appeared to arise from an endogenous substance as it was present in blank extractions, especially when the urine had been stored for an extended period. Its presence, however, did not effect the screen in any way.

Volunteer samples

Thirty volunteers, working in pairs were given one of the fifteen diuretics at the lowest recommended dose, and spot urine specimens were collected at 4, 8

and 24 h post-dose for screening. These results are summarized in Table IV.

Sensitivity study

A 2-ml urine specimen was spiked to contain one twentieth of the amount used in the reference standard, the final concentrations being 1.5 μg/ml chlorothiazide, mefruside and bumetanide; 1.0 μg/ml chlorthalidone, frusemide and metolazone; and 0.5 μg/ml hydrochlorothiazide, quinethazone, methyclothiazide, bendrofluazide and cyclopenthiazide. A separate sample containing 1.0 μg/ml clopamide was also prepared. These specimens were extracted, reonstituted in 100 μl of 35% acetonitrile in 0.01 mol/l phosphate buffer (pH 3.0) and 5 μl were injected. At an integrator sensitivity setting of 0.032 a.u.f.s., all twelve peaks obtained were at least 9% of full scale deflection (Table I).

DISCUSSION

We were prompted by a number of requests to our laboratory and by evidence in the published literature [8–15] regarding the surreptitious use of diuretics, to develop a reliable screening procedure for potassium-depleting diuretics available in Australia. Using the described procedure we can simply and reliably screen a random urine sample for twelve of the fifteen potassium-depleting diuretics studied. The three diuretics not included in the screen are indapamide, clorexalone and ethacrynic acid even though they could be chromatographically separated from the others with retention times of 5.8, 6.0 and 8.3 min, respectively. These three drugs are extensively metabolised. Only 5–7.3% of a dose of indapamide is excreted as unchanged drug in the urine [16, 17], therefore making it difficult to detect as part of a general screen. If analytical conditions are optimized for indapamide it could be detected individually as shown by Pietta et al. [18].

A single study of Corbett et al. [19] has shown that clorexalone is totally metabolised via oxidative monohydroxylations into three major products which are excreted in the urine. In the present study, there was no evidence of these metabolites in the urinary extracts from two volunteers, both of whom had taken 10 mg of clorexolone.

Ethacrynic acid is primarily excreted into bile with 50% or more of the drug appearing in faeces [20, 21]. Beyer et al. [21] reported that about 6–14% of unchanged ethacrynic acid was excreted in the urine of dogs. By using optimal extraction and chromatographic conditions we were able to detect ethacrynic acid down to levels of 0.3 μg/ml in urine to which the drug had been added. There was, however, no detectable ethacrynic acid peak in the urine extracts from two volunteers who had each taken 50 mg ethacrynic acid and had urine specimens collected at 4, 8 and 24 h after the dose. Stüber et al. [22] described the use of gas chromatography–mass spectrometry for the determination of ethacrynic acid in plasma but have only worked with spiked samples. This method could be tried bearing in mind that the elimination half-life of ethacrynic acid is only 0.5–1.0 h.

For the twelve potassium-depleting diuretics included in the screen, sample preparation time is minimized as the urine is extracted directly, without the need for any reduction or derivatization steps. This is an advantage over the method

of Tisdall et al. [7] which cannot detect chlorothiazide directly and requires reduction of chlorothiazide to hydrochlorothiazide, prior to extraction. Consequently this necessitates a second sample preparation without the reduction step to ensure that hydrochlorothiazide itself is not present. If the clean-up wash with phosphate buffer pH 7.5 is omitted, a chromatogram with several extraneous peaks results. This wash step is responsible for the low recovery figures for chlorothiazide and frusemide. We have found, however, that the large dosage of chlorothiazide and the sensitivity of our screen compensate adequately to enable us to detect chlorothiazide levels as low as 1 μg/ml in urine. Similarly, the loss of frusemide in the wash step did not cause any detection problems in the patient and volunteer studies as levels down to 0.4 μg/ml in urine were easily detected.

It should be noted that methyclothiazide (retention time, t_R 4.68 min) and clopamide (t_R 4.72 min) are not resolved and if a diode array detector is not available to obtain an ultraviolet spectrum of a peak in this region, it would have to be reported as either methyclothiazide or clopamide.

For the detetion of cyclopenthiazide the sensitivity must be increased four-fold by either adjusting the sensitivity of detection, reconstitution volume, injection volume or volume of urine extracted. We have found that plotting a post-run chromatogram at an altered integrator sensitivity setting (e.g. 0.016 a.u.f.s.) or alternatively monitoring the chromatogram at a wavelength of 230 nm is the simplest way of detecting cyclopenthiazide which is present in relatively small concentrations due to the low dose normally used.

The evaluation of the screen using normal volunteers showed twelve diuretics were detectable up to 24 h after a low dose. The inability to detect indapamide, clorexalone and ethacrynic acid does not greatly reduce the overall value of the screen considering that sale of these three drugs together represent less than 1.5% of the Australian diuretic market; individual figures estimated by the respective manufacturers are: indapamide, $<1.0\%$; clorexalone, $<0.1\%$; and ethacrynic acid tablets, $<0.4\%$. We therefore conclude that the screen would be reliable for the detection of commonly used diuretics and will therefore be of clinical use.

REFERENCES

1 M. Ahnoff, M. Ervik, P. Lagerström, B.-A. Persson and J. Vessman, J. Chromatogr., 340 (1985) 73.
2 S.J. Stohs and G.A. Scratchley, J. Chromatogr., 114 (1975) 329.
3 I.L. Honigberg, J.T. Stewart, A.P. Smith and D.W. Hester, J. Pharm. Sci., 64 (1975) 1201.
4 P.J. Smith and T.S. Hermann, Anal. Biochem., 22 (1968) 134.
5 V.B. Pilsbury and J.V. Jackson, J. Pharm. Pharmacol., 18 (1966) 713.
6 D. Sohn, J. Simon, M.A. Hanna, G. Ghali and R. Tolba, J. Chromatogr., 87 (1973) 570.
7 P.A. Tisdall, T.P. Moyer and J.P. Anhalt, Clin. Chem., 26 (1980) 702.
8 A. Ando, H. Koshida, T. Morise, I. Miyamori, R. Miyazaki, I. Koni, J. Sudo, N. Funaki, Y. Tofuku, M. Kuroda, et al., Nippon Jinzo Gakkai Shi, 27 (1985) 519.
9 M. Lopez Jimenez, F.J. Barbado, F. Mateos, J.M. Pena, A. Gil, F. Arnalich, I. Tovar, F.G. Alonso and J.J. Vazquez Rodriguez, Med. Clin. (Barcelona), 84 (1985) 23.
10 H. Kaufmann, F. Elijovich and M.D. Yahr, Mt. Sinai J. Med. N.Y., 51 (1984) 625.
11 V. Di Giacomo and A. Tedeschi, Clin. Ter. 112 (1985) 163.
12 H. Marty, Schweiz. Med. Wochenschr., 115 (1985) 250.

13 I. Rubinstein, Ann. Emerg. Med., 14 (1985) 275.
14 J.E. Mitchell, D. Hatsukami, E.D. Eckert and R.L. Pyle, Am. J. Psychiatry, 142 (1985) 482.
15 R.A. Sansone, Am. Fam. Physician, 30 (1985) 141.
16 D.B. Campbell and E.M. Phillips, Eur. J. Clin. Pharmacol., 7 (1974) 407.
17 L.J. Klunk, S. Ringel and E.S. Neiss, J. Clin. Pharmacol., 23 (1983) 377.
18 P. Pietta, A. Calatroni and A. Rava, J. Chromatogr., 228 (1982) 377
19 K. Corbett, S.A. Edwards, G.E. Lee and T.L. Threlfall, Nature (London), 208 (1965) 286.
20 C.D. Klaassen and T.J. Fitzgerald, J. Pharmacol. Exp. Ther., 191 (1974) 548.
21 K.H. Beyer, J.E. Baer, J.K. Michaelson and H.F. Russo, J. Pharmacol. Exp. Ther., 147 (1965) 1.
22 W. Stüber, E. Mutschler and D. Steinbach, J. Chromatogr., 227 (1982) 193.