Journal of Chromatography, 310 (1984) 179—187
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2173

MASS SPECTROMETRIC DETERMINATION OF N-HYDROXYPHENACETIN IN URINE USING MULTIPLE METASTABLE PEAK MONITORING FOLLOWING THIN-LAYER CHROMATOGRAPHY

NOEL W. DAVIES, MAURICE E. VERONESE and STUART McLEAN*

School of Pharmacy and Central Science Laboratory, University of Tasmania, P.O. Box 252C, Hobart, Tasmania 7001 (Australia)

(First received January 26th, 1984; revised manuscript received April 4th, 1984)

SUMMARY

This work describes a method for the quantitative determination of the labile, toxic N-hydroxy metabolite of phenacetin in urine. A thin-layer chromatography step was used for the preliminary purification of extracts, and the specificity of the assay was based on the monitoring of specific metastable decompositions in a forward geometry double-focussing mass spectrometer, in a manner analogous to conventional tandem mass spectrometry. This precluded the need for a gas chromatographic separation, thus minimizing thermal decomposition which can occur with these compounds, as well as enabling very rapid analyses.

INTRODUCTION

The toxicity of the antipyretic—analgesic drug, phenacetin, is considered to be at least partly mediated by its N-hydroxy metabolite [1]. The formation of N-hydroxyphenacetin (NHP), and its biochemical reactivity, have been studied extensively in vitro, but little is known of the extent of N-hydroxylation of phenacetin in vivo. The problem has been to measure NHP, which is only formed in small amounts from phenacetin, in the presence of other phenacetin metabolites and endogenous interfering substances in urine. A variety of assays have been reported for NHP and similar N-arylacetylhydroxamic acids. At first, colorimetric methods were used for urinary analysis [2], but these lack specificity [3]. NHP and other N-arylacetylhydroxamic acids formed in biochemical reactions in vitro have been assayed by high-performance liquid chromatography (HPLC) [4, 5], gas chromatography—mass spectrometry (GC—MS) [6], and thin-layer chromatography (TLC) using radiolabelled phenacetin [7]. We have previously reported the assay of urinary NHP by GC

0378-4347/84/$03.00

of its methylated derivative, N-methoxyphenacetin (NMP), using flame-ionization detection [8]. However, as mentioned in that report [8], NMP can undergo decomposition under GC conditions, so we sought a more reliable assay of NHP.

This paper describes the determination of NHP, as NMP, using TLC to separate NMP from most interfering substances, followed by specific detection by MS using metastable peak monitoring. This MS technique deserves some introductory remarks.

Increasing use is being made of mass spectrometers with more than one analysing region to act as specific and sensitive detectors for target compounds in relatively impure samples. Much of this work has been carried out with reversed geometry [9, 10] or tandem quadrupole [11, 12] instruments. In these cases, the two sectors are used sequentially, the first to isolate an ion of interest and the second to examine its metastable or collisionally induced decomposition products (MS—MS). Full daughter ion spectra [13, 14] or selected daughter ion monitoring [15—17] have been used. Double-focussing mass spectrometers of normal geometry have also been used, in which the metastable or collisionally induced decompositions occur in the first field-free region, just after the ion source. This was used originally to increase the specificity of GC—MS [18, 19], and more recently has been applied to direct probe analyses [20, 21]. Although the sectors are not used sequentially in normal geometry instruments, since the products of specific decompositions are being monitored, it nevertheless constitutes a form of MS—MS.

EXPERIMENTAL

Materials

NHP was prepared and characterised as previously described [8]. As internal standard, the deuterated analogue of N-hydroxyphenacetin was synthesized from 4-nitro[2,3,5,6-$^2H_4$]phenol (KOR Isotopes, Cambridge, MA, U.S.A.). The deuterated 4-nitrophenol was first ethylated, using ethyl iodide—potassium carbonate in acetone, after the method of Vogel [22]. The resulting 4-nitro-[$^2H_4$]phenetole was reduced and selectively acetylated to give N-hydroxy-[2,3,5,6-$^2H_4$]phenacetin (DNHP), by the method used for the synthesis of unlabelled NHP [8]. DNHP was identical to NHP in m.p. (104°C) and TLC mobility, and on reduction with titanous chloride it yielded a single product which co-chromatographed with phenacetin on TLC [8]. The mass spectrum of DNHP was similar to that of NHP [23] except the major fragments were four a.m.u. higher. The isotopic purity was 98% $^2H_4$.

N-Butyryl-4-aminobenzoic acid (4-BABA) was prepared by reacting 4-aminobenzoic acid with butyric anhydride, and was recrystallized from 95% ethanol, m.p. 228—231°C. 4-BABA ran as a single substance on TLC, and its methylated derivative, N-butyryl-4-aminomethyl benzoate (4-BAMB), behaved as one compound on TLC and GC. The mass spectrum of 4-BAMB was consistent with its structure.

Diazomethane was made in small quantities, fresh as required, from *p*-tosylsulphonylmethylnitrosamide [22], and was used as the ethereal solution. Other chemicals and solvents were of analytical grade.

Extract of *Helix pomatia* (β-glucuronidase plus arylsulphatase) was obtained from Boehringer (Mannheim, F.R.G.).

Apparatus

Glass TLC plates (20 × 20 cm) were coated with silica gel (0.25-mm thick) containing a fluorescent marker (Sigma type GF, size 10—40 μm; St. Louis, MO, U.S.A.). The mass spectrometer was a Vacuum Generators (U.K.) 70/70F double-focussing instrument, with a digital scan controller, linked-scan unit, mode switch, 8-channel M.I.D. unit and 2035 data system with magnet switching selected ion software. The instrument was operated in the electron impact mode at 70 eV and 4 kV main beam accelerating voltage.

Extraction and methylation

Urine was collected from subjects who had taken a dose of phenacetin (10 mg/kg in two gelatin capsules), and samples were kept at −20° C until analysed. The analysis was commenced on the day of collection, since the glucuronide conjugate of NHP is known to be unstable in aqueous solution [24].

Calibration curves were obtained by addition of known amounts of NHP (in methanol) to urine from an undosed subject.

A 2-ml aliquot of urine was placed in a 30-ml stoppered centrifuge tube, 200 μl of 1.10 *M* acetate buffer, pH 5.2 were added and the final solution was adjusted to pH 5.2, if necessary, with 5 *M* hydrochloric acid. To this were added 100 μl extract of *Helix pomatia* and 25 μl of methanol containing the internal standard. The internal standard was originally 25 μg of 4-BABA, but eventually 5 μg of DNHP were used when DNHP became available. The mixture was incubated overnight (17 h) at 37° C to hydrolyse conjugated NHP.

The tubes were then cooled in ice, the hydrolysate adjusted to pH 1.0 with 5 *M* hydrochloric acid (200 μl) and extracted with 15 ml methylene chloride by vortexing for 30 sec. The phases were separated by centrifugation (1200 *g*, 5 min), and freezing (dry ice—acetone), and the methylene chloride decanted into a 50-ml round bottomed flask. The extract was taken to dryness on a rotary evaporator (40° C), the residue redissolved in 2 ml methanol, and cooled in ice. Methylation was achieved with 2 ml ethereal diazomethane (1 h, on ice) and the reaction mixture again evaporated to dryness.

Thin-layer chromatography

The residue in the flask was redissolved in 200 μl methylene chloride and applied as a 5-cm strip to a silica gel TLC plate, which was developed twice with chloroform. A band which moved with reference NMP (R_F 0.26) was scraped off and eluted by vortexing for 30 sec with 10 ml methanol. After sedimenting the silica gel by centrifugation (1200 *g*, 5 min) the methanol was transferred by Pasteur pipette to a 50-ml round bottom flask, and the solvent removed with a rotary evaporator. The residue was redissolved in 200 μl methylene chloride and transferred to a conical-tipped tube ready for analysis.

Mass spectrometry

The daughter ions chosen for monitoring NMP and DNMP were *m/z* 135

and 139, respectively (see Results). The two metastable peaks were alternately brought to focus by a combination of linked magnetic field/electric sector (B/E) and accelerating voltage switching, through a modification of a previously described method [21].

At a resolution of 1000, the ion at *m/z* 214 in the mass spectrum of perfluorotributylamine was selected as the starting point of a theoretical B/E linked scan (i.e. the magnetic field which transmitted *m/z* 214 was linked to the electric sector voltage). B/E scans from *m/z* 209 and 213 could then be easily focussed by small accelerating voltage adjustments [25]. Accelerating voltages $V_1 = (209/214) \times V_0$ and $V_2 = (213/214) \times V_0$, where V_0 was the accelerating voltage at which the main beam was being transmitted, were entered into two channels of the M.I.D. unit, the output of which was routed directly to the accelerating voltage programmed power supply, bypassing the normal electric sector coupling. The appropriate constant parent ion scans were then focussed when the respective channel was selected.

Two genuine daughter ion channels were entered, at *m/z* 85.16 ($= 135^2/214$) (channel 2), and *m/z* 90.28 ($= 139^2/214$) (channel 4) into the magnetic selected ion software, along with two additional channels at *m/z* 82.66 ($= 133^2/214$) (channel 1) and *m/z* 87.70 ($= 137^2/214$) (channel 3), with the linked scan unit remaining on. These channels resulted in *m/z* 133, 135, 137 and 139 daughter ions being selected, and to be shown as such on the mass indicator of the 70/70.

A small sample of standard NMP and DNMP was loaded onto the direct insertion probe, and using a small sweep of the B/E scan over 1 mass unit each genuine daughter channel was accurately centred with the appropriate M.I.D. channel selected. The collector slit was then opened to give flat topped peaks.

The accelerating voltage was cycled to switch during daughter channels 1 and 3 so that the appropriate voltage was selected when genuine daughter channels 2 and 4 were selected. Dwell time was 1 sec for each accelerating voltage channel, and 500 msec, including reset time, for each daughter ion channel, with a full cycle resulting every 2 sec. For synchronization, the accelerating voltage cycle was started and the magnet switching was then initiated at the appropriate instant to result in V_1 being selected during channel 1 and V_2 during channel 3.

To avoid a very rapid distillation of NMP from the probe, a relatively cool (120°C) source temperature was used. This resulted in the distillation maximizing at about 20 sec after insertion of the probe, providing 20 sampling points.

A 1-μl aliquot of the sample was loaded on to the direct insertion probe and the results of the 209 → 135 and 213 → 139 decompositions were acquired in the manner described, being displayed as intensity versus time. The area of each was measured on the data system and the ratio calculated for determination of unknown concentrations.

RESULTS AND DISCUSSION

Thin-layer chromatography

Preliminary experiments showed that the extracted hydrolysate contained

too many interfering substances for NHP to be determined by direct insertion MS—MS, as we had previously found it to be too impure for GC analysis [8]. TLC was used to separate NHP from most of these interfering compounds.

Methylation of NHP gave a stable derivative for TLC. NHP itself tails badly on silica gel [8], possibly because of chelation to trace metals [4]. Hinson and Mitchell [7] found it necessary to add non-radioactive NHP to minimize adsorption losses of [^{3}H]NHP during their TLC assay.

Recovery from urine

Blank urine was spiked with NHP to examine the effect of pH on the extraction of NHP into methylene chloride. In this experiment 4-methylacetanilide was used to standardize the GC assay [8]. Recovery of NHP was better at pH 1 (95%) than at pH 3 or 5 (65%).

The methylation reaction mixture was analysed at various times up to 24 h to check on the completeness of NHP conversion to NMP. The reaction was 90% complete in 30 min, and no further NMP was formed after 1 h.

Optimal conditions for hydrolysis of conjugated NHP were found by treating urine from a subject dosed with phenacetin with different amounts of enzyme and various incubation times. It was found that 0.1 ml extract *Helix pomatia* per 2 ml urine gave the highest recovery of NHP, as well as most phenolic metabolites of phenacetin (to be reported elsewhere). An exception was 2-hydroxyphenacetin (2HP), whose recovery, and therefore hydrolysis, was maximal with 0.04 ml enzyme mixture. Incremental additions of enzyme (0.02 ml) resulted in a progressive decline in 2HP recovery, and a concomittant increase in NHP found. It seems likely that this reciprocal relationship is due to two competing reactions: the rearrangement of NHP-glucuronide to 2HP-glucuronide [24] and the enzymatic hydrolysis of the NHP-glucuronide. More enzyme, and therefore a faster hydrolysis, leaves less time for the rearrangement to occur. Lacking an NHP-glucuronide standard, we were unable to check on the extent of residual isomerization under conditions giving maximum recovery of NHP.

Using 0.1 ml enzyme, hydrolysis was found to be complete after incubation for 17 h (overnight).

Mean overall recovery was 63%, estimated from the NMP/DNMP ratio found when 5 μg NHP were added to urine and 5 μg DNHP added at the methylation step.

Mass spectrometry

Fig. 1 shows the normal 70-eV mass spectrum of NMP, with that of DNMP, in which all the major ions are shifted by 4 a.m.u. A full B/E linked scan from the molecular ion (*m/z* 209) of NMP, to find the direct daughter ions from the first field-free region, gave a spectrum with *m/z* 179 (2.5%), 178 (5%), 167 (100%), 166 (10%), and 135 (18%) as the only significant peaks. The ions at *m/z* 167 and 166 corresponded to the loss of ketene and an acetyl radical respectively, and were also found to occur in the normal and B/E spectra of the isomeric ring methoxylated compounds, 2- and 3-methoxyphenacetin [23]. Therefore these daughter ions were not suitable for monitoring NMP in the presence of isomeric phenolic metabolites of phenacetin. However, the

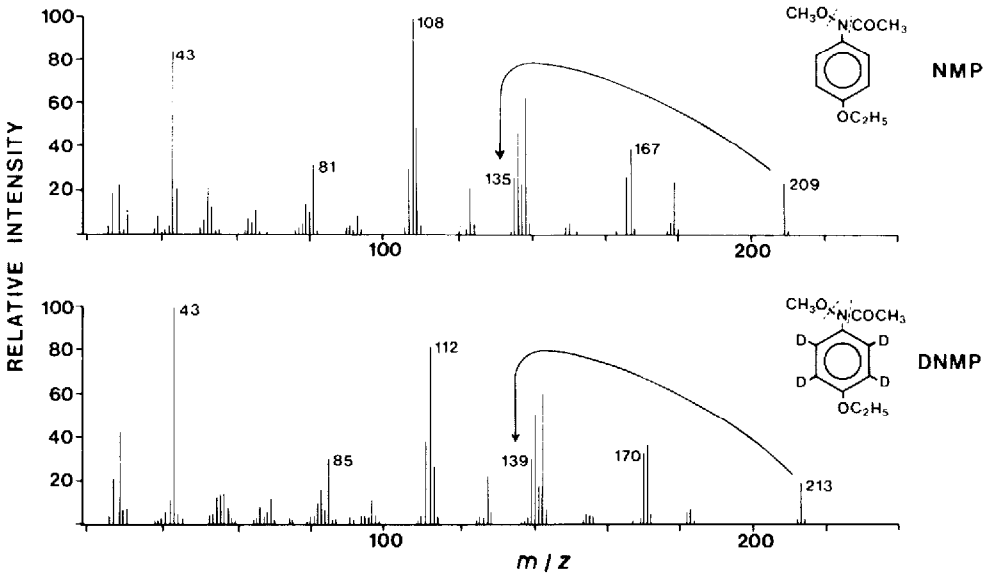

Fig. 1. Electron impact mass spectra of NMP and DNMP, with arrows showing the metastable reactions monitored for quantitative analysis. Broken lines show the sites of cleavage in these reactions.

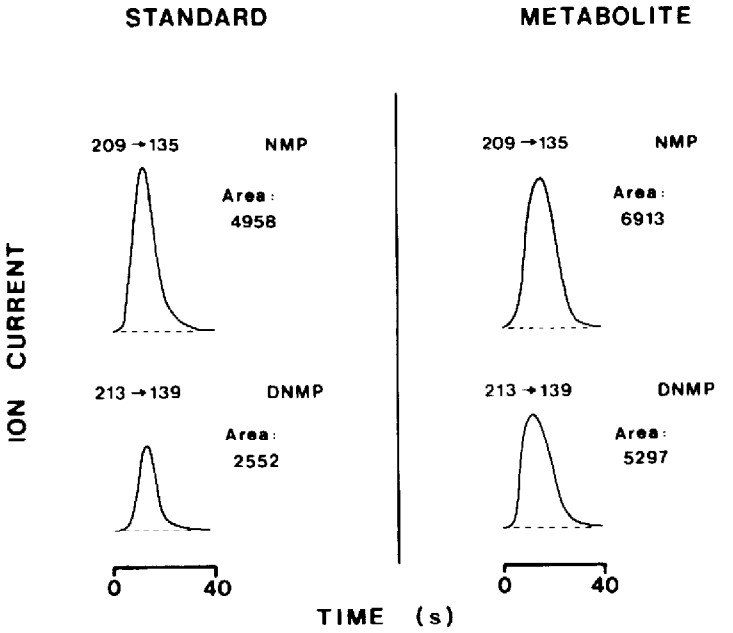

Fig. 2. Ion traces obtained during metastable peak monitoring of NMP and DNMP. The left side shows a calibration standard, in which 10 μg NHP was added to 2 ml blank urine. The right side shows analysis of urine following a dose of phenacetin; 6.7 μg NHP was found per 2 ml urine.

daughter ions at *m/z* 179, 178 and 135 were not found to be present in the normal or B/E spectra of the ring-substituted compounds, making them suitable candidates for a specific assay. Due to the relative size, and specificity of the loss, the ion at *m/z* 135 was chosen as being the most suitable. This ion was found from the high-resolution studies of the main beam *m/z* 135 ion to be C_8H_9NO, corresponding to the loss of the elements of the acetyl and methoxy groups by an undetermined mechanism. Thus the decompositions monitored were 209 → 135 for NMP, and 213 → 139 for DNMP, as indicated in Figs. 1 and 2.

An extract of hydrolysed urine from an undosed subject, without any preliminary purification, was monitored for these decompositions to determine the extent, if any, of interference from compounds other than NMP. This indicated there was some interference from other 209 → 135 decompositions, or additional reactions which could be focussed under the conditions employed [21]. Although the interference did not display the distillation profile of NMP from the direct insertion probe, it would nevertheless have made accurate quantification difficult. Hence a preliminary purification was necessary, and the TLC procedure was employed. An examination of the extract of a TLC scrape at the R_F of NMP from an undosed subject indicated virtually no detectable signal in either channel being monitored.

Calibration curve

Addition of NHP (1—20 μg) and DNHP (5 μg) to 2 ml blank urine gave a good linear relationship using either peak heights or peak areas (ratio NMP/DNMP versus amount NHP added). Peak areas gave a slope of 0.191, *y*-intercept 0.03, and correlation coefficient 0.9983. Peak heights gave a slope of 0.186, *y*-intercept 0.055, and correlation coefficient 0.9994. Repeated measurements on the same sample gave an instrumental coefficient of variation (C.V.) of 1.94% (n = 8). Replicate assays using eight lots of 2 ml urine from one urine collection sample from a subject who had taken phenacetin gave a C.V. of 9.2% (by peak area) and 7.2% (by peak height).

The selectivity of the assay depends on two separation steps: (1) TLC and (2) the selection of specific daughter ions produced from the molecular ions of NMP and DNMP in the first field-free region of the mass spectrometer. Thus NHP can be assayed in the presence of many other metabolites of phenacetin which are mostly formed in far greater amounts.

In early experiments, before DNHP was available, 4-BABA was used successfully as an internal standard. In this case the metastable reaction monitored was 221 → 151 (the molecular ion losing C_4H_6O). There was no interference from blank urine, and the calibration curve was linear (slope 0.0098, *y*-intercept 0.0018, correlation coefficient 0.9993). The only unsatisfactory aspect of 4-BAMB was that it distilled off the probe much more slowly than NMP, which considerably prolonged the MS analysis time. DNMP, having the same distillation profile as NMP, enabled much faster analyses, and is the ideal internal standard.

Formation of NHP from phenacetin in man

Fig. 3 shows the urinary excretion of NHP by a male subject following a

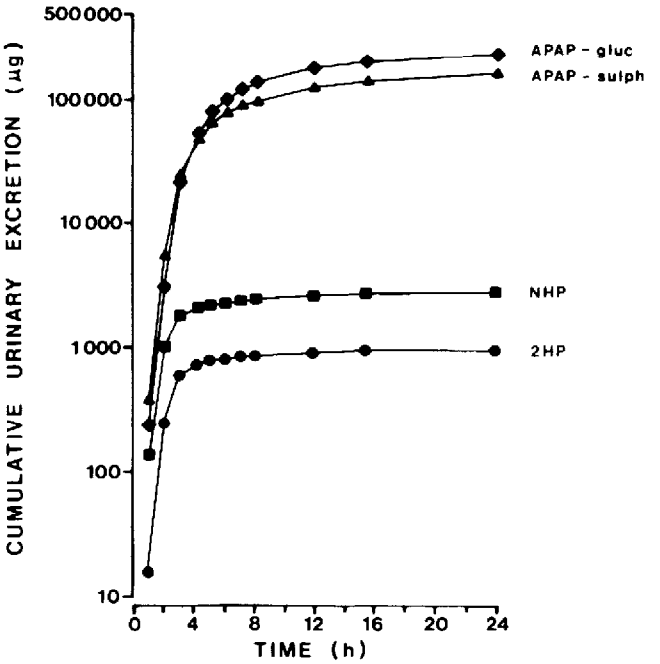

Fig. 3. Cumulative urinary excretion of NHP and some other phenacetin metabolites, following an oral dose of 10 mg/kg phenacetin in a male subject. APAP-gluc (♦) is paracetamol glucuronide and APAP-sulph (▲) is paracetamol sulphate. These two major metabolites of phenacetin are included for comparison with NHP (■) and 2HP (●), and their HPLC determination will be described in a separate communication.

dose of phenacetin (10 mg/kg, orally in two gelatin capsules). The presence of NHP in urine was separately confirmed by GC—MS, as previously described [8]. The total amount of NHP excreted in 24 h corresponded to 0.40% of the dose of phenacetin. This is comparable with the finding of 0.28% NHP after 900 mg phenacetin, in which NHP was determined by its ability to chelate copper and extract it into water [2]. However, in view of the complex metabolism of phenacetin, we believe that our structurally specific assay is preferable.

The assay is sensitive to less than 0.5 µg NHP per ml urine, which appears quite adequate for metabolic studies involving urine analysis.

ACKNOWLEDGEMENTS

We are grateful to Mrs. Heather Galloway for technical assistance and for drawing the figures, and to Miss Helen Lawler for typing the manuscript. We are particularly grateful to Mr. M. Power for the modification of the mass spectrometer electric sector coupling.

REFERENCES

1 J.R. Gillette, S.D. Nelson, G.J. Mulder, D.J. Jollow, J.R. Mitchell, L.R. Pohl and J.A. Hinson, in R. Snyder, D.V. Parke, J.J. Kocsis, D.J. Jollow, C.G. Gibson and C.M. Witmer (Editors), Biological Reactive Intermediates - II, Plenum, New York, 1982, p. 931.

2 S. Belman, W. Troll, G. Teebor and F. Mukai, Cancer Res., 28 (1968) 535.
3 J.H. Weisburger and E.K. Weisburger, Pharmacol. Rev., 25 (1973) 2.
4 M.D. Corbett and B.R. Chipko, Anal. Biochem., 98 (1979) 169.
5 J.A. Hinson, L.R. Pohl and J.R. Gillette, Anal. Biochem., 101 (1980) 462.
6 I.M. Kapetanovic, J.S. Dutcher and J.M. Strong, Anal. Chem., 49 (1977) 1843.
7 J.A. Hinson and J.R. Mitchell, Drug Metab. Dispos., 4 (1976) 430.
8 S. McLean, N.W. Davies, H. Watson, W.A. Favretto and J.C. Bignall, Drug Metab. Dispos., 9 (1981) 255.
9 R.W. Kondrat and R.G. Cooks, Anal. Chem., 50 (1978) 81A.
10 F.W. McLafferty, Acc. Chem. Res., 13 (1980) 34.
11 R.A. Yost and C.G. Enke, Anal. Chem., 51 (1979) 1251A.
12 R.A. Yost, H.O. Brotherton and R.J. Perchalski, Int. J. Mass Spectrom. Ion Phys., 48 (1983) 77.
13 G.A. McLuskey, R.G. Cooks and A.M. Knevel, Tetrahedron Lett., 46 (1978) 4471.
14 J. Henion, G.A. Maylin and B.A. Thomson, J. Chromatogr., 271 (1983) 107.
15 R.W. Kondrat, G.A. McLuskey and R.G. Cooks, Anal. Chem., 50 (1978) 2017.
16 M. Youssefi, R.G. Cooks and J.L. McLaughlin, J. Amer. Chem. Soc., 101 (1979) 3400.
17 P. Prome, C. Lacave, J. Roussel and J.C. Prome, Biomed. Mass Spectrom., 9 (1982) 527.
18 S.J. Gaskell and D.S. Millington, Biomed. Mass Spectrom., 5 (1978) 557.
19 S.J. Gaskell, R.W. Finney and M.E. Harper, Biomed. Mass Spectrom., 6 (1979) 113.
20 E.K. Chess and M.L. Gross, Anal. Chem., 52 (1980) 2057.
21 N.W. Davies, J.C. Bignall and M.S. Roberts, Biomed. Mass Spectrom. , 10 (1983) 646.
22 A.I. Vogel, Practical Organic Chemistry, Longmans, London, 1956.
23 N.W. Davies, W. Lenk and S. McLean, Org. Mass Spectrom., 17 (1982) 649.
24 G.J. Mulder, J.A. Hinson and J.R. Gillette, Biochem. Pharmacol., 27 (1978) 1641.
25 N.W. Davies, J.C. Bignall and R.W. Lincolne, Org. Mass Spectrom., 17 (1982) 451.