Journal of Chromatography, 414 (1987) 139–148
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 3427

APPLICATION OF MOLECULAR-SECONDARY-ION MASS SPECTROMETRY FOR DRUG METABOLISM STUDIES

I. DIRECT ANALYSIS OF CONJUGATES BY THIN-LAYER CHROMATOGRAPHY–SECONDARY-ION MASS SPECTROMETRY

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(First received July 1st, 1986; revised manuscript received September 11th, 1986)

SUMMARY

Molecular-secondary-ion mass spectrometry (SIMS) is a suitable method for the analysis of non-volatile substances such as conjugated metabolites of drugs. We have developed a simple method for the direct SIMS measurement of conjugates following thin-layer chromatography without any extraction procedure. After separation with a butanol–acetic acid–ethanol–water (3:1:1:1, v/v) system, the spot was cut out and attached to a SIMS probe. The conjugates of *p*-nitrophenol and 4-hydroxyantipyrine were measured. The quantitative application of the method is also discussed, using deuterium-labelled internal standards for *p*-nitrophenol conjugates.

INTRODUCTION

Conjugated metabolites resulting from phase II metabolism of xenobiotics are the ultimate excretory products from in vivo detoxification of foreign compounds. Recently, enzymes concerning the phase II reaction have been shown to form unstable products which are carcinogenic, strongly inflammatory and necrotic to the digestive and excretory organs [1]. Determination of the extent of conjugation reactions for each drug is important for understanding its disposition and toxicopharmacological effects. The most widely used method for the analysis of conjugates by conventional mass spectrometry (MS) involves several procedures, i.e. chemical or enzymatic hydrolysis to obtain aglycones and their derivatization to volatiles. These indirect methods are troublesome and sometimes time-consuming. The results from non-specific hydrolysis may lead to erroneous structural assigments of the original conjugated form.

Molecular-secondary-ion mass spectrometry (SIMS) and fast atom bombard-

0378-4347/87/$03.50

ment (FAB) are recognized as powerful soft ionization techniques for the analysis of non-volatile organic substances. Two different separation techniques have been reported in connection with the use of SIMS and FAB, i.e. high-performance liquid chromatography (HPLC) [2,3] and thin-layer chromatography (TLC) [4–6]. TLC is an efficient, simple and low-cost separation technique. The direct measurement of conjugates by SIMS is possible by simply cutting out the TLC spot and attaching it to the SIMS probe.

We have succeeded in using TLC–SIMS for the identification and quantitation of conjugated metabolites of *p*-nitrophenol (PNP) and 4-hydroxyantipyrine (4OHA) in the negative-ion mode.

EXPERIMENTAL

Chemicals

p-Nitrophenyl-β-D-glucuronide (PNPG) and *p*-nitrophenyl sulphate (PNPS) were purchased from Sigma (St. Louis, MO, U.S.A.). Deuterium-labelled PNPG-d_4 and PNPS-d_4, used as internal standards, were synthesized according to the method of Halac, Jr. and Reff [7] and Watanabe et al. [8] using PNP-d_4 (99 atom % ^2H; from MSD Isotopes, Montreal, Canada) as a starting material. 4OHA was obtained from Aldrich (Milwaukee, WI, U.S.A.). Aluminium-backed TLC sheets, with silica gel 60 F_{254}, were obtained from Merck (Darmstadt, F.R.G.). All solvents and chemicals used were of analytical grade and were purchased from Wako (Osaka, Japan).

Preparation of 4-hydroxyantipyrine glucuronide (4OHAG) and sulphate (4OHAS)

4OHAG and 4OHAS were synthesized enzymatically according to the following method. Isolated hepatocytes were prepared from male Wistar rats (250–270 g), as described by Moldéus et al. [9]. 4OHA was incubated with $2 \cdot 10^6$ cells per ml at an initial concentration of 0.1 m*M*, at 37°C for 2 h in Krebs–Henseleit buffer solution (pH 7.4) under a carbon dioxide–oxygen stream (5:95). After incubation, the cells were separated by centrifugation for 20 min at 50 *g*. The supernatant was freeze-dried. Then the dried material was reconstituted with 90% methanol for the TLC development.

Animal study

Male Wistar rats (250–270 g) were administered a single dose of 100 mg/kg PNP intravenously. Urine was collected for the next 24 h. The urine sample was diluted to 50 ml with water. Both 75 μg of PNPG-d_4 and 15 μg of PNPS-d_4 were added to 1 ml of urine. The solution was freeze-dried and the residue was reconstituted with 500 μl of 90% methanol. After centrifugation, the supernatant was brought onto the TLC plate.

Calibration

Calibration curves for PNPG and PNPS were obtained by plotting the ratio of the ion intensity of PNPG-d_0/PNPG-d_4 and PNPS-d_0/PNPS-d_4. Non-labelled

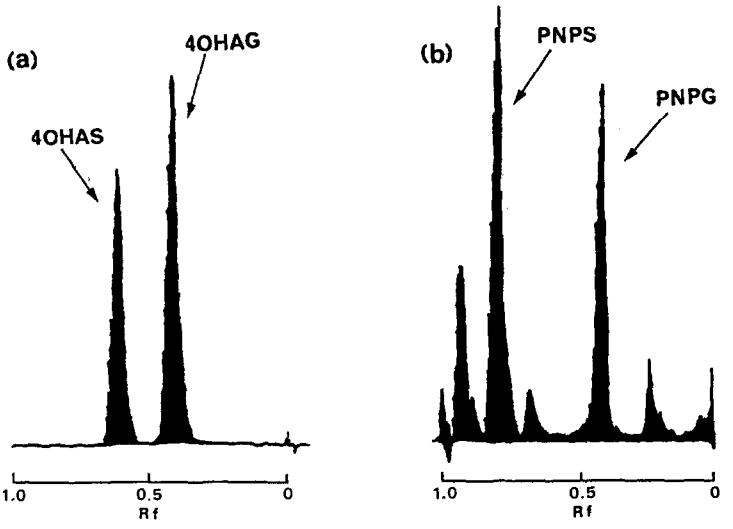

Fig. 1. TLC densitograms of (a) 4OHA conjugates from isolated hepatocytes and (b) PNP conjugates from urine.

PNPG (0–200 μg) was added to 75 μg of PNPG-d_4. PNPS-d_4 (15 μg) was added to 0–100 μg of PNPS. Then each standard solution was brought onto the TLC plate.

TLC development

The PNPG-, PNPS-, 4OHAG- and 4OHAS-containing samples were separated on a silica gel plate using *n*-butanol–acetic acid–ethanol–water (3:1:1:1, v/v). A precise microsyringe (Microliter No. 7001, Hamilton, Reno, NV, U.S.A.) was used for applying the samples. The spots were detected by a short-wavelength UV lamp or a UV densitometric method using a Chromatoscanner CS-910 (Shimadzu, Kyoto, Japan). The area of interest on the plate (ca. 5×10 mm) was cut out and was attached to a SIMS probe tip using a strip of double-faced masking tape.

SIMS measurement

A Hitachi M-80A high-resolution mass spectrometer equipped with a SIMS source and an M-003 data processing system (Hitachi, Tokyo, Japan) was used. The operation conditions and procedures were as follows.

(1) The primary Xe^+ and secondary-ion accelerating voltage were set at 8 and 3 kV, respectively.

(2) The TLC tip was inserted manually after addition of 10–15 μl of glycerol or thioglycerol as the matrix liquid.

(3) Both positive- and negative-ion mass spectra were measured for structure elucidation and for quantitation purposes.

(4) The characteristic fragment ions were monitored by repeated scanning and

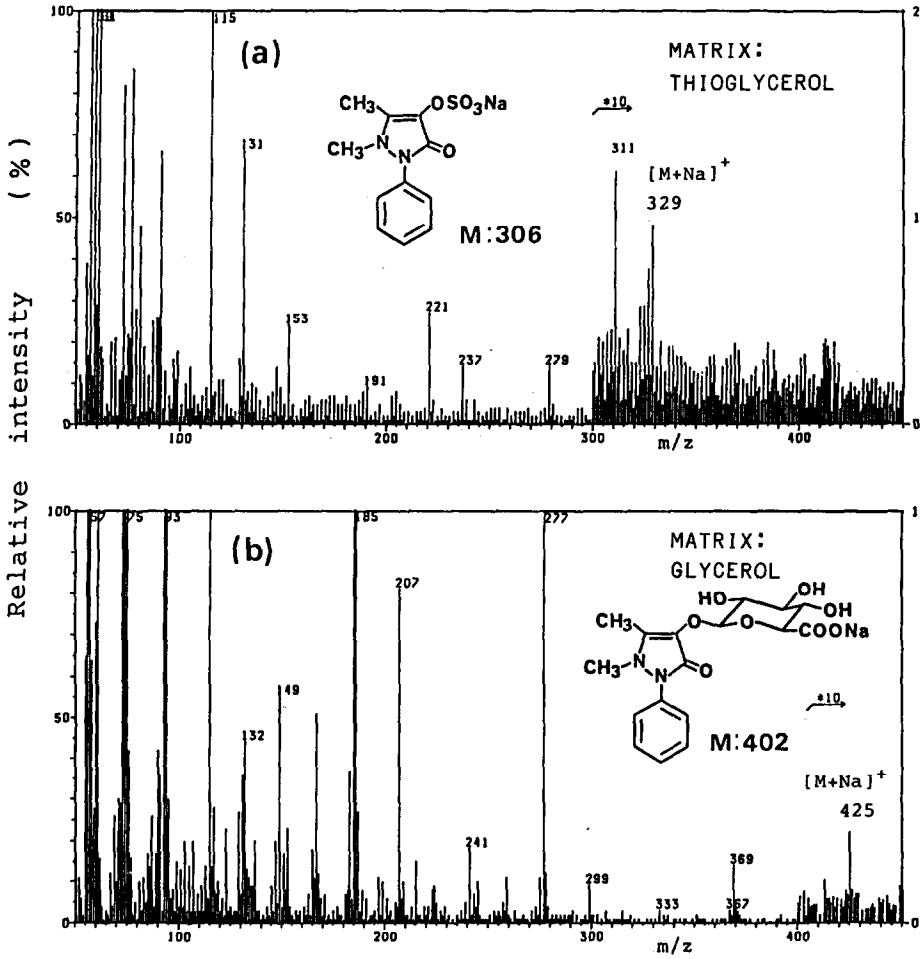

Fig. 2. TLC-positive-SIMS spectrum of (a) 4OHAS and (b) 4OHAG.

the mean peak intensities during every ten scans (m/z 1–400) were used for the quantitation.

RESULTS AND DISCUSSION

Characterization of 4OHAG and 4OHAS

4OHA, one of the main metabolites of antipyrine (AP), is found in the urine of both rat and man. We have reported [10] that the conjugated metabolites of 4OHA are excreted in higher amounts by smokers and "smoking" rats compared with non-smoking control groups. These findings may be attributed to an alteration of the hepatic mono-oxygenase system by smoking. However, we could identify neither 4OHAG nor 4OHAS directly without the aid of specific enzyme hydrolysis in the previous study. Two major products were formed in almost equal amounts in the isolated rat hepatocytes system, when 4OHA was used as a sub-

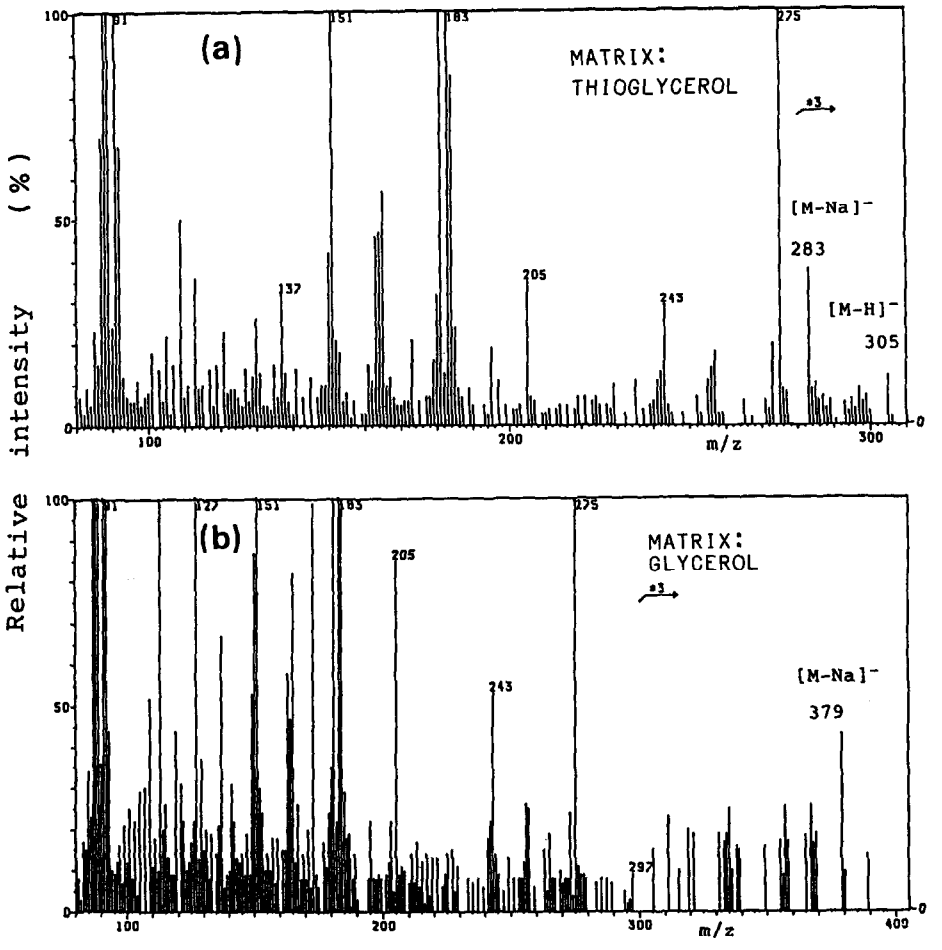

Fig. 3. TLC-negative-SIMS spectrum of (a) 4OHAS and (b) 4OHAG.

strate as shown in the profiles of the TLC densitometric assay (Fig. 1b). The higher R_F value spot (0.6) was checked first by TLC–SIMS. The positive- and negative-ion mass spectra are shown in Fig. 2a and 3a , respectively. From the positive-ion spectrum, *m/z* 329 and 307 could be attributed to $[M+Na]^+$ and $[M+H]^+$ of 4OHAS. In addition, *m/z* 305, 283 and 97 ions in the negative-ion spectrum could be attributed to $[M-H]^-$, $[M-Na]^-$ and $[SO_4H]^-$ of 4OHAS, respectively. The lower spot (R_F 0.4) was found to be 4OHAG from the mass spectra shown in Fig. 2b and 3b: $[M+Na]^+$ (*m/z* 425), $[M-Na]^-$ (379) and [M−glucuronate]$^-$ (203). Moreover this spot coloured purple when treated with resorcinol reagent. The negative-ion spectra were clearer than those of the positive ions in both cases.

TLC-SIMS spectrum of PNPG and PNPS

PNPS (R_F=0.8) and PNPG (R_F=0.4) were separated satisfactorily with TLC using the same conditions as for the 4OHA conjugates. Both negative- and posi-

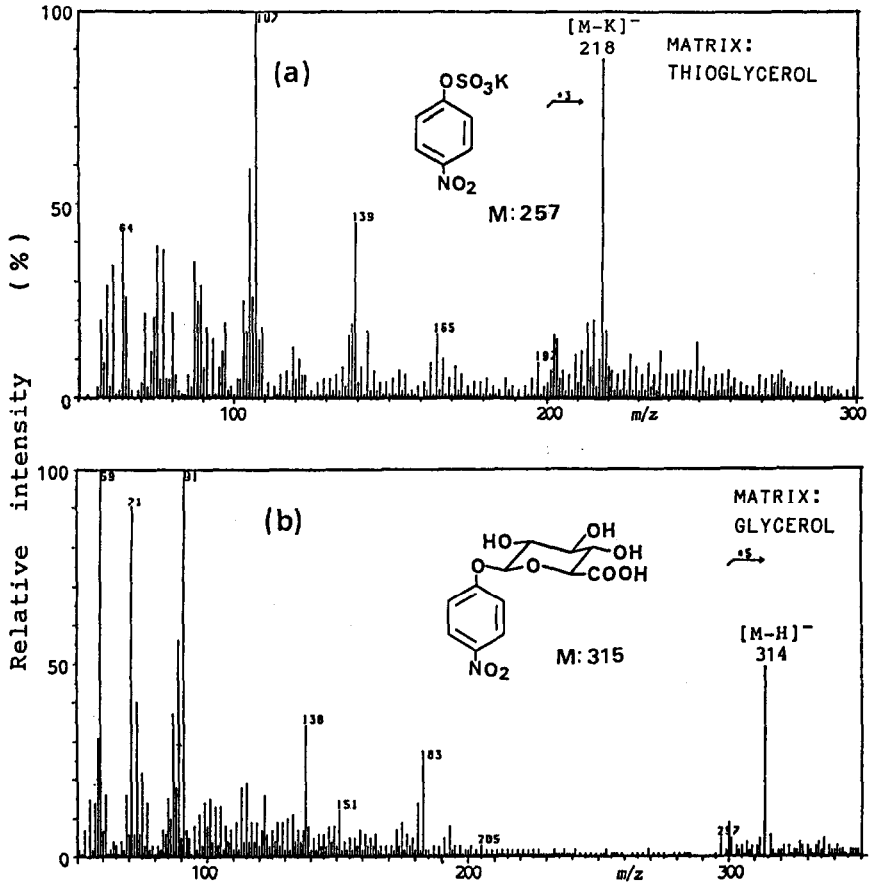

Fig. 4. TLC-negative-SIMS spectrum of (a) PNPS and (b) PNPG.

tive-ion spectra for PNPS and PNPG were measured using the normal SIMS holder (Ag plate). The negative-ion spectra gave a better signal-to-noise ratio than the positive-ion spectra, as was also the case for the 4OHAG conjugates. The negative SIMS spectra for PNPS and PNPG on TLC plate are shown in Fig. 4a and b. More intense spectra were obtained compared with those of 4OHAS and 4OHAG. PNPS and PNPS-d_4 gave an abundant $[M-X]^-$ ion (*m/z* 218 and 222), where X is H or other alkaline metal and the *m/z* 218 ion is $[O_2N-C_6H_4-O-SO_3]^-$. PNPG and PNPG-d_4 yielded an $[M-H]^-$ ion at *m/z* 314 and 318. The *m/z* 138 ion which corresponds to the aglycone moiety ($[O_2N-C_6H_4-O]^-$) was observed with PNPG. The lower limit to obtain a spectrum of each conjugate with a satisfactory signal-to-noise ratio was found to be 2 μg per spot.

Calibration curves

The suitability of TLC–SIMS for the quantitation of sulphate and glucuronide conjugates was evaluated with respect to the sensitivity and to the selectivity

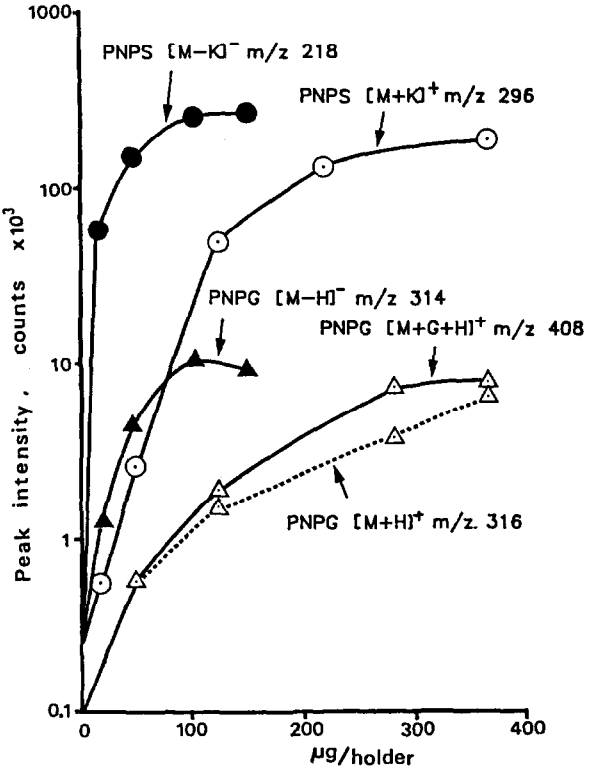

Fig. 5. Relationship between peak intensity and amount of sample with negative- and positive-SIMS.

between the negative- and positive-ion detection, using a regular type SIMS holder. The amount of PNPG and PNPS ranged from 0 to 350 μg per holder. The ions for the quantitation were chosen as follows: $[M+K]^+$ (296) and $[M-K]^-$ (218) for PNPS; $[M+H]^+$ (316) and $[M-H]^-$ (314) for PNPG. As shown in Fig. 5, the negative ions $[M-K]^-$ and $[M-H]^-$ gave steeper curves when the ion intensity was plotted against the amount of sample on the holder compared with the positive ions. The same conclusion was obtained when these conjugates were measured on a TLC plate.

The calibration curves obtained from the absolute measurement of negative ions for PNPS and PNPG were compared with a stable isotope dilution method. In the latter method, the following ions were selected, *m/z* 218 for PNPS, 222 for PNPS-d_4, 314 for PNPG and 318 for PNPG-d_4. The total peak intensity increased linearly after twenty scans for PNPG. For PNPS, the total peak intensity increased until twenty scans were recorded. The ratio d_0/d_4 was constant [coefficient of variation (C.V.) $<5\%$] both with PNPS and PNPG for 10–40 or 10–80 scans as shown in Fig. 6. These characteristic tendencies were affected by the matrix, i.e. glycerol and thioglycerol. Glycerol presented a more constant ionization than did thioglycerol. However, the sensitivity when using thioglycerol was about ten times higher than with glycerol in case of PNPS measurement. A mixture would be more suitable as a general-purpose matrix. In both compounds the

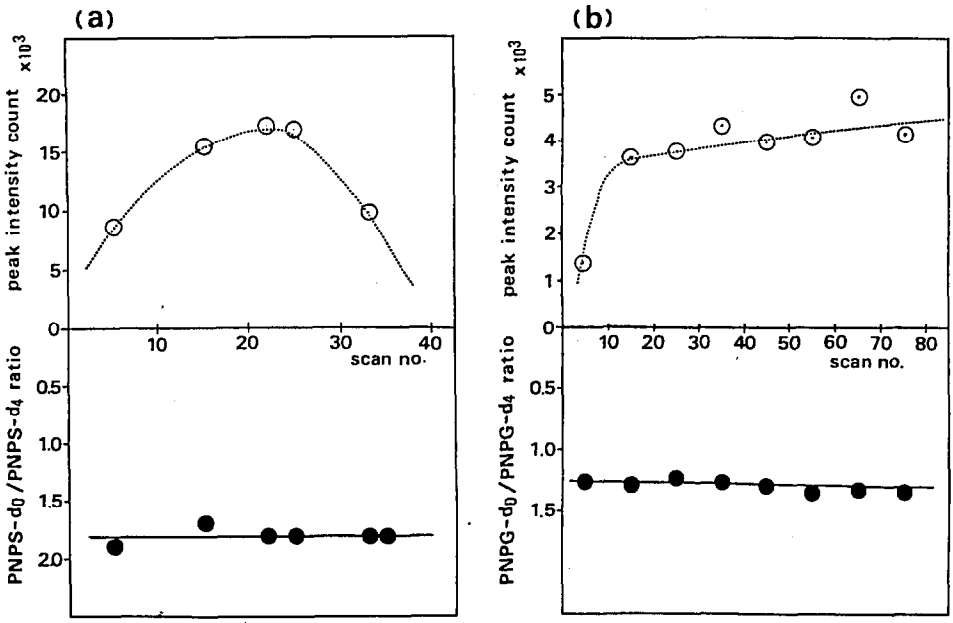

Fig. 6. Stability of ionization for (a) PNPS and (b) PNPG with an absolute method or a stable isotope dilution method by TLC–negative-SIMS.

TABLE I

URINARY EXCRETION OF PNPS IN RATS AFTER INTRAVENOUS ADMINISTRATION OF PNP

Rat No.	TLC-negative-SIMS		HPLC	
	mg	% of dose	mg	% of dose
1	4.08	22.07	3.24	17.54
2	5.92	29.80	5.04	25.34
3	9.07	47.87	8.24	43.49
4	9.27	46.65	9.37	47.17
Mean ± S.E.	7.09 ± 1.26	36.60 ± 6.36	6.47 ± 1.42	30.70 ± 6.11

scan was repeated 10–40 times and the mean ratios for the intensity of d_0 to d_4 were calculated. Then the linear calibration curves for the amount of PNPS-d_0 and PNPG-d_0 were calculated from the intensities by the least-squares method. Satisfactory linearities (correlation coefficients, r) were obtained both for PNPS ($r=0.999$) and PNPG ($r=0.998$). However, in each case the line did not pass through the origin which was attributed to isotopic impurities in the d_4-labelled standard and to background contamination for the d_0-channel.

Urine analysis

The results from the TLC–SIMS analysis for the urinary excretion of PNPS by rats compared with those from HPLC analysis [11] are listed in Table I. The

mean value obtained from TLC–SIMS corresponds with that from HPLC. In rat Nos. 1 and 2, the lower excretion group of PNPS, the unchanged PNP and PNPG were higher than in the higher excretion group (Nos. 3 and 4). These individual differences are an interesting phenomena. Whether these differences were the result of intrinsic factors or experimental fluctuation should be resolved in a further study. The amount of PNPG excreted was determined by the TLC–SIMS method only in one case (rat No. 4) to be 15.1% (HPLC: 21.5%). In other cases, a biological contaminant from urine disturbed the quantitation. Special clean-up procedures, such as two-dimensional TLC, HPLC and other column chromatography, may be necessary to minimize the contributions from endogenous contamination.

CONCLUSION

The applicability of TLC for the separation and clean-up before MS was described by Danigel et al. [12]. They compared TLC with HPLC–MS for the analysis of etoposide in human plasma. One advantage is the lower cost of the material consumed during sample preparation; HPLC is ten times more expensive than TLC. Nearly twenty samples could be analyzed per hour in their TLC–MS system, whereas in HPLC–MS only one sample can be analyzed per hour. The superiority of TLC–MS in efficiency will also be true for our system, if we can actually compare HPLC–SIMS (or –MS) with TLC–SIMS.

TLC–SIMS is easy to handle and reliable. It will become a more popular technique by the application of an automatic sample scanning method, and it will be an effective tool in the field of life science.

ACKNOWLEDGEMENTS

We are thankful to Dr. David L. Hachey, Children's Nutrition Research Center, Baylor College of Medicine, Houston, TX, U.S.A., for his helpful discussion on this study. We would like to thank Dr. Hideyo Shindo, director of our laboratories, for his encouragement throughout the work.

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