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Analysis of unconjugated morphine, codeine, normorphine and morphine as glucuronides in small volumes of plasma from children

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Abstract: A sensitive method for the analysis of unconjugated morphine, codeine, normorphine and total morphine after hydrolysis of glucuronide conjugates is described. The method was applicable to 50-μl volumes of plasma. The analytes were converted to heptafluorobutyryl (HFB) derivatives before analysis by gas chromatography–negative ion chemical ionization mass spectrometry. Morphine and codeine were quantified against their [$^2H_3$]-isotopomers. Linearity, precision and accuracy were quite acceptable (in the 10^{-10}–10^{-9} g range), and the absolute limits of detection were <1 pg.

Keywords: *GC–MS; morphine; morphine glucuronides; codeine; normorphine.*

Introduction

Post-operative relief of pain in children has been carried out using opioids in a continuous infusion for a number of years [1]. The subcutaneous route for delivery of the drug has been used for many years in the terminal care of adults is subcutaneous delivery [2] and this route is an attractive means of delivering the drug to children since it avoids intramuscular injections or the conventional continuous infusion, where access to, and the preservation of, small veins may be difficult [3]. Pharmacokinetic data for this means of delivering morphine has been obtained for adults [4] but not for children. In order to develop this type of protocol more fully, and assess the efficacy of the procedure it is necessary to obtain data on the concentrations of morphine and its metabolites in plasma following subcutaneous administration to children. The samples of blood that can be obtained from children during a pharmacokinetic study are necessarily small and limited by ethical constraints. Thus a sensitive and discriminating method was required in order to make multiple measurements of morphine and its metabolites in such small volumes of plasma.

Morphine is converted in the liver to its glucuronide metabolites and most of the morphine present in plasma, particularly after oral dosing, is in the form of its 3-glucuronide (M3G). The 6-glucuronide of morphine (M6G) is a potent analgesic [5, 6] and may also be present in plasma; at *ca* 10% of the concentration of M3G [5]. The amount of M3G in plasma following oral dosing is 10–50 times the amount of morphine, and thus M6G [6, 7] may be present in plasma in greater amounts than morphine.

A number of methods based on HPLC or GC–MS have been developed for the analysis of morphine [8–14]. Fewer methods have been developed for the analysis of the glucuronide metabolites of morphine, and the existing methods for the measurement of these compounds based on HPLC [7–11] are difficult to use and lack sensitivity; more sensitive methods have to be used for small volumes of samples. Recently, an elegant refinement of radioimmunoassay (RIA) technique has been described which was able to determine both morphine and M6G by using different antisera [15]. The method was validated using an established HPLC method. GC–MS methods cannot compete with RIA methods for speed but they are more specific than either HPLC or RIA methods.

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In this paper we describe a very sensitive and specific GC–MS method for measuring unconjugated morphine, codeine, normorphine and morphine as glucuronide(s) in small volumes of plasma from children.

Experimental

Chemicals

All solvents used were HPLC grade (Rathburn Chemicals, Peebleshire, UK). Chemicals and standards were obtained from the following sources: morphine sulphate pentahydrate, codeine free base, [$^2H_3$] morphine, [$^2H_3$] codeine, normorphine hydrochloride dihydrate, morphine 3-glucuronide, morphine 6-glucuronide dihydrate, glucuronidases from *Escherichia coli*, bovine liver, *Helix pomatia*, *Patella vulgata* and *Chlamys opercularis* (the Sigma Chemical Co., Dorset, UK); heptafluorobutyric anhydride (Aldrich Chemical Co., Dorset, UK).

Plasma samples

Morphine was infused subcutaneously at rates between 5 and 25 $\mu g\ kg^{-1}\ h^{-1}$. Samples of blood (1 ml) were collected at 4-h intervals from children receiving subcutaneous morphine infusion up to a total of 10 ml. The samples (up to a total of 1 ml kg^{-1} of body weight or 10 ml maximum regardless of body weight) were collected into a lithium heparin tube and then centrifuged, the plasma removed and the sample stored at −20°C until analysis.

Treatment of samples

The volume of plasma obtained from 1 ml of blood was *ca* 0.5 ml. An aliquot of sample (300 µl) was withdrawn and to this were added 15 ng of [$^2H_3$] morphine (15 µl of a 1 ng μl^{-1} solution in water) and 15 ng of [$^2H_3$] codeine (15 µl of a 1 ng μl^{-1} solution in water).

(i) In order to determine unconjugated morphine in the spiked plasma sample two aliquots (each of 55 µl) were withdrawn and phosphate buffer (55 µl, 0.1 M, pH 6.8) was added to each, this was followed by addition of ammonia buffer (300 µl, 1 M, pH 9.5) and extraction with ethyl acetate (2 × 1 ml). The ethyl acetate was then removed under a stream of nitrogen and heptafluorobutyric anhydride (50 µl) was added to the residue. The solution was heated (15 min, 60°C), the reagent was then removed under a stream of nitrogen, the residue was dissolved in ethyl acetate (100 µl) and 4 µl were injected into the GC–MS.

(ii) In order to determine 3MG and 6MG the remaining plasma (220 µl) was mixed with phosphate buffer (220 µl, 0.1 M, pH 6.8) containing *E. coli* glucuronidase (2 mg ml^{-1}) and the mixture was then incubated at 42°C for 18 h. Two aliquots of 110-µl were withdrawn and treated as described in (i) starting from ". . . followed by addition of ammonia buffer . . .".

Calibration curve

The following solutions of standards were prepared

(a) [$^2H_3$] morphine, [$^2H_3$] codeine (each 1 ng μl^{-1}).

(b) Morphine, codeine and normorphine (0.05 ng μl^{-1}) + M3G and M6G (each 0.1 ng μl^{-1}).

(c) Morphine, codeine and normorphine (each 0.2 ng μl^{-1}) + M3G and M6G (each 0.4 ng μl^{-1}).

(d) Morphine, codeine and normorphine (each 0.8 ng μl^{-1}) + M3G and M6G (each 1.6 ng μl^{-1}).

(e) Morphine, codeine and normorphine (each 3.2 ng μl^{-1}).

Samples of plasma (300 µl) were spiked as follows;

(i) 15 µl of solution (a) + 15 µl of water;

(ii) 15 µl of solution (a) + 15 µl of solution (b);

(iii) 15 µl of solution (a) + 15 µl of solution (c);

(iv) 15 µl of solution (a) + 15 µl of solution (d);

(v) 15 µl of solution (a) + 15 µl of solution (e).

The spiked plasma samples (330 µl) were then treated as described under *Treatment of samples*.

Replicate precision

The following solutions of standards were prepared;

(a) [$^2H_3$] morphine and [$^2H_3$] codeine (each of 1 ng μl^{-1});

(b) Morphine, codeine and normorphine (each of 0.2 ng μl^{-1}) and M6G and M3G (each of 0.4 ng μl^{-1}).

A sample of blank plasma (1 ml) was spiked with 50 µl of solution (a) and 50 µl of solution (b) and mixed thoroughly.

(i) Five aliquots (each of 55 µl) were withdrawn and processed according to *Treatment of samples* (i).

(ii) The remaining plasma (0.825 ml) was mixed with phosphate buffer (0.825 ml, 0.1 M, pH 6.8) containing 2 mg ml^{-1} glucuronidase and the mixture was incubated at 42°C for 18 h. Five aliquots (each of 110 μl) were withdrawn and processed as previously aliquots described under *Treatment of samples.*

Instrumentation

A Hewlett–Packard 5998A GC–MS system was used. Analysis was carried out in the negative ion chemical ionization (NICI) mode. Methane was introduced to give a source pressure of *ca* 1 Torr. The GC was fitted with a Hewlett–Packard HP-1 column (12 m × 0.25 mm i.d. × 0.33 μm film), helium was used as a carrier gas with a head pressure of 5 p.s.i. The GC injector temperature was 250°C and the interface temperature was 280°C. The oven was programmed as follows: 100°C (1 min)/20°C min^{-1} to 250°C and then 2°C min^{-1} to 257°C. The mass spectrometer was tuned to the ions for the PFTBA calibrant at *m/z* 452, 595 and 633.

Results and Discussion

Figure 1 shows the mass spectrum of morphine di HFB derivative obtained under NICI conditions. The principal ions are the molecular ion at *m/z* 657, an ion at *m/z* 637 (resulting from loss of HF from the molecular ion), an ion at *m/z* 441 (due to a less straightforward fragmentation, probably involving addition of hydrogen followed by loss of HF and HFB) and a reagent specific ion (at *m/z* 197) which is the base peak. Selected ion monitoring was carried out for the ions at *m/z* 657 and *m/z* 637 in the analysis of morphine $[^2H_3]$ morphine di HFB gave corresponding ions with the addition 3 a.m.u. HFB derivatives of normorphine and codeine also gave simple mass spectra under NICI conditions. Table 1 summarises the mass spectral and retention index data obtained for morphine and related compounds. Figure 2 shows an SIM trace for the HFB derivatives of a mixture of morphine, codeine and normorphine spiked at 0.38, 0.5 and 0.42 ng per 50 μl of plasma (the concentrations are expressed in terms of the free bases), respectively. The trace represents injection of *ca* 20 pg of each compound on column and these are compared with $[^2H_3]$ morphine and $[^2H_3]$ codeine spiked at 2.5 ng per 50 μl of plasma (*ca* 100 pg of each on column). The absolute limits of detection of the HFB derivatives of these compounds were below 1 pg.

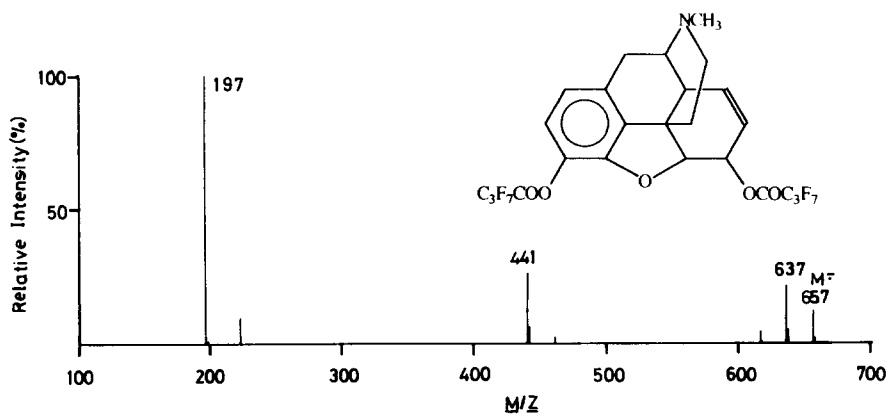

Figure 1
The mass spectrum of morphine diHFB derivative under NICI conditions.

Table 1
Mass spectral and chromatographic data

Compound	Base peak	M^-	Other major ions	*I* value
Morphine	197	657 (12.9)	637 (21.5), 441 (26.8)	2333
$[^2H_3]$ morphine	214	660 (14.1)	640 (22.3), 444 (34.6)	2332
Codeine	475	475 (100)	213 (88.5)	2338
$[^2H_3]$ codeine	478	478 (100)	213 (85.7)	2336
Normorphine	623	839 (7.8)	603 (34.7), 213 (56.8)	2404

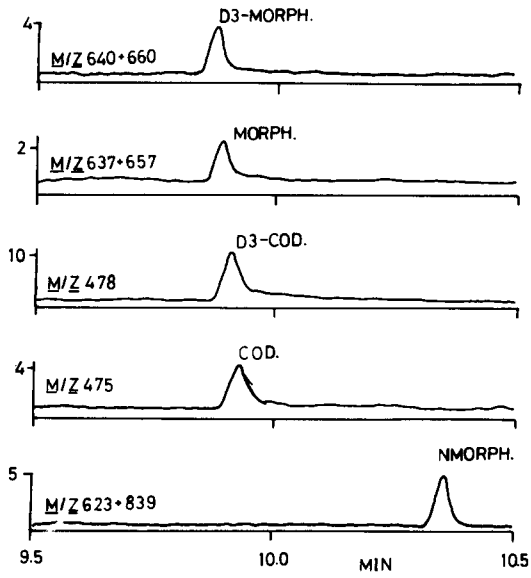

Figure 2
SIM trace showing the HFB derivatives of morphine, codeine and normorphine (0.2 ng per sample) in comparison with [$^2H_3$] morphine and codeine (2.5 ng per sample).

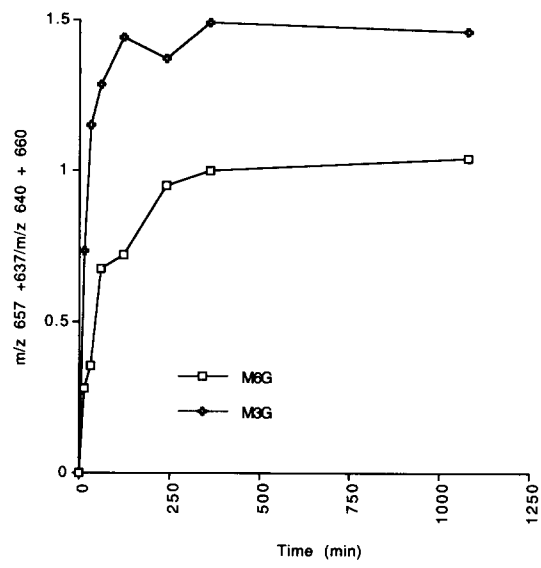

Figure 3
Release of morphine from M3G and M6G incubated with glucuronidase from *E. coli*.

Glucuronidases from *H. pomatia*, *P. vulgata*, *C. opercularis*, bovine liver and *E. coli* were tested for their ability to hydrolyse M3G and M6G and it was found that the *E. coli* glucuronidase was the most effective. The other enzymes gave <10% hydrolysis of the more readily hydrolysed M3G after 30 min. The *H. pomatia* enzyme has been used by a number of workers [16–18] and an early paper on its use [16] would seem to indicate that it only gives about 10% hydrolysis of M3G after 1 h whereas in our case the *E. coli* enzyme gave >80%. Acid hydrolysis has been commonly used to release morphine from its conjugates [14, 19], but here it was found that even mild acid hydrolysis led to extensive degradation. Typical curves for the hydrolysis of M3G and M6G by the *E. coli* glucuronidase are shown in Fig. 3. The hydrolysis of M3G was *ca* 70% complete in 30 min, whereas the hydrolysis of M6G was only *ca* 27% compete at this time. In principle, this provides a means of distinguishing between the two glucuronides but, in practice, the fact that M3G is 5–10 times more abundant that M6G in plasma [5] means that such a distinction is not possible within the limits of precision of the method.

The estimates for morphine as glucuronide are thus based on the curve constructed using morphine sulphate as a standard rather than curves constructed for the glucuronides. The reason for this was that the commercial glucuronides were not pure as judged from the peak area for the morphine released after 18 h of hydrolysis compared to a calibration curve based on morphine sulphate, and the purities of M3G and M6G can be estimated as being *ca* 78 and 64%, respectively. It is not clear whether or not these standards have been fully authenticated and it is likely that they contain undefined amounts of absorbed water or exist in the form of more than one hydrate.

Calibration curves were constructed for morphine, codeine and normorphine by monitoring the selected ions for the compounds and deuteriated internal standards given in Table 2. Normorphine was quantified against [$^2H_3$] morphine. The curves were constructed with respect to the compounds as their free bases and were linear over the following ranges: morphine 0.095–6.08 ng per 50 μl of plasma; codeine 0.125–8.0 ng per 50 μl of plasma; and normorphine 0.105–6.72 ng per 50 μl of plasma.

The precision and accuracy for the analysis of five samples of morphine (0.38 ng per 50 μl of plasma), codeine (0.5 ng per 50 μl of plasma) and normorphine (0.42 ng per 50 μl of plasma) was determined, and is shown in Table 3. The accuracy of the method was determined by comparing the amount of morphine, codeine or normorphine calculated from the

Table 2
Calibration curve details for morphine, codeine and normorphine

Compound	Ion ratio used to prepare curve	Equation of line	Corr. coeff.	Range ng per 50 µl
Morphine	$\frac{m/z\ 637 + 657}{m/z\ 640 + 660}$	$y = 1.2522x - 0.0143$	0.9998	0.095–6.08
Codeine	$\frac{m/z\ 475}{m/z\ 478}$	$y = 0.7044x - 0.0170$	0.9914	0.125–8.0
Normorphine	$\frac{m/z\ 839 + 623}{m/z\ 640 + 660}$	$y = 1.2536x - 0.2360$	0.9964	0.105–6.72

Table 3
Precision and accuracy of morphine, codeine and normofphine in subnanogram amounts

Compound	Amt spiked per 50 µl plasma	Accuracy $n = 5$	Precision $n = 5$
Morphine	0.38 ng	97.1%	±10.6%
M3G + M6G	0.8 ng each	—	±14.6%*
Codeine	0.5 ng	94.0%	±8.8%
Normorphine	0.42 ng	111.9%	±11.8%

*Determined after incubation for 18 h with glucuronidase.

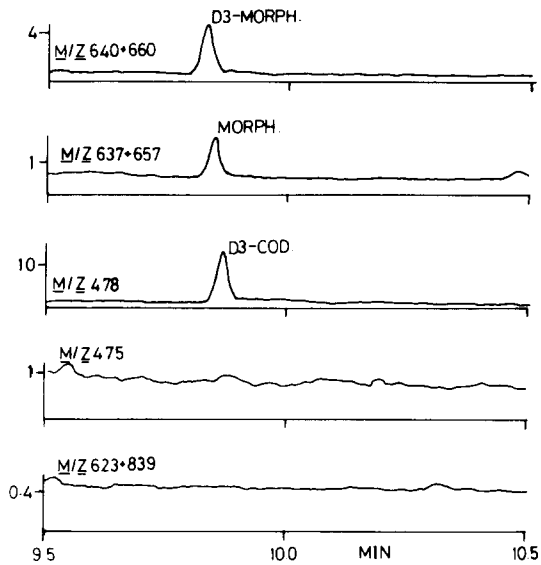

Figure 4
SIM trace showing morphine in a sample of plasma (50 µl) from a child receiving morphine via subcutaneous infusion for 24 h. Of [$^2H_3$] morphine and codeine, 2.5 ng were added as internal standards.

analytical data using the calibration curve in comparison with the known amount of these compounds spiked into the sample. The precision of the analysis of M3G + M6G (0.8 ng per 50 µl of plasma) after incubation with glucuronidase for 18 h was determined, it was not possible to determine the accuracy in view of the doubts about the purity of the standards. The precision was quite good for determination of compounds at this level, the main source of imprecision was the difficulty of getting exactly reproducible integration with respect to the chromatographic baseline.

Figure 4 shows derivatized unconjugated morphine extracted from plasma (50 µl) following subcutaneous infusion of morphine for 24 h into a child comparison with standard derivatized [$^2H_3$] morphine (2.5 ng per 50 µl). Figure 5 shows the same sample after treatment with glucuronidase for 18 h, indicating a large increase in the peak for morphine. After hydrolysis of the plasma samples with glucuronidase, it was possible to detect small amounts of normorphine. In some samples small amounts of codeine were detected at levels close to the limit of detection. Table 4 shows some preliminary results obtained after the analysis of plasma following the continuous

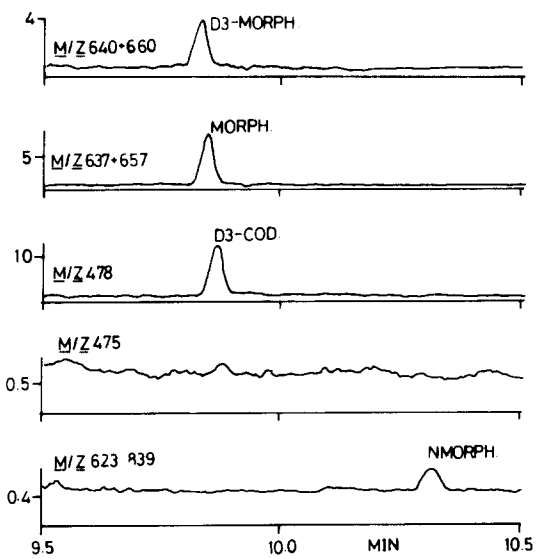

Figure 5
SIM trace showing morphine and normorphine in a sample of plasma (50 µl) from a child receiving morphine via subcutaneous infusion for 24 h. After hydrolysis with glucuronidase. Of [$^2H_3$] morphine and codeine, 2.5 ng were added as internal standards.

Table 4
The variation of the concentration of unconjugated and total morphine and total normorphine with time of subcutaneous infusion of morphine into children*

	Patient 1			Patient 2†		
Time (h)	Unconj. morphine (ng ml^{-1})	Total morphine (ng ml^{-1})	Total normorph. (ng ml^{-1})	Unconj. morphine (ng ml^{-1})	Total morphine (ng ml^{-1})	Total normorph. (ng ml^{-1})
0	4.72 ± 0.50	12.97 ± 0.47	ND‡	6.53	17.48	ND
4	4.48 ± 0.17	14.4 ± 0.76	ND	6.35	18.33	ND
8	—	—	—	3.82	13.77	ND
14	4.94 ± 0.21	39.78 ± 2.52	1.00 ± 0.50	—	—	—
18	6.73 ± 0.71	49.65 ± 0.72	0.89 ± 0.07	—	—	—
22	6.35 ± 0.10	58.1 ± 1.50	0.96 ± 0.12	5.74	41.18	1.37
24	5.24 ± 0.57	47.97 ± 0.88	0.96 ± 0.06	5.78 ± 0.24	47.18 ± 6.64	2.48 ± 0.72
26	5.44 ± 0.47	44.79 ± 2.89	0.88 ± 0.65	6.64	54.72	2.82
28	7.52 ± 0.32	58.54 ± 2.20	1.30 ± 0.23	5.62 ± 0.3	49.32 ± 6.48	2.67 ±0.85

*Codeine was below the limit of detection (*ca* 0.25 ng ml^{-1}) in the 50-μl samples analysed.
†Some single measurements were made on samples from this patient.
‡Not detected.

subcutaneous infusion of morphine (5–25 μg kg^{-1} h^{-1}) into children.

References

[1] S.E.F. Jones and M.A. Stokes, *Anaesthesia* **46**, 688–690 (1991).
[2] T.A. Goudie, M.W. Allan, M. Lonsdale, L.M. Burrow, W.A. Macrae and I.S. Grant, *Anaesthesia* **40**, 1086–1092 (1985).
[3] R. McNicol, *Br. J. Anaesth.* **71**, 752–756 (1993).
[4] C.S. Waldmann, J.R. Eason, E. Rambohul and G.C. Hanson, *Anaesthesia* **39**, 768–771 (1984).
[5] J. Sawe, J.O. Svensson and A. Rane, *Br. J. Clin. Pharmacol.* **16**, 85–93 (1983).
[6] R. Osbourne, S. Joel, D. Trew and M. Slevin, *Lancet* **I**, 828 (1988).
[7] R. Osbourne, P. Thompson, S. Joel, D. Trew, N. Patel and M. Slevin, *Br. J. Clin. Pharmacol.* **27**, 499–505 (1989).
[8] J.O. Svensson, A. Rane, J. Sawe and F. Sjoquist, *J. Chromatogr.* **230**, 427–432 (1982).
[9] J.O. Svensson, *J. Chromatogr.* **375** 174–178 (1986).
[10] P. Joel, R.J. Osborne and M. Slevin, *J. Chromatogr.* **430**, 394–399, (1988).
[11] R.F. Venn and A. Michalkiewicz, *J. Chromatogr.* **525**, 379–388 (1990).
[12] J.L. Mason, S.P. Ashmore and A.R. Aitkenhead, *J. Chromatogr.* **570**, 191–197 (1991).
[13] R.H. Drost, R.D. Van Ooijen, T. Ionescu and R.A.A. Maes, *J. Chromatogr.* **310**, 193–198 (1984).
[14] A.W. Jones, Y. Blom, U. Bondesson and E. Anggard, *J. Chromatogr.* **309**, 73–80 (1984).
[15] D.J. Chapman, S.P. Joel and G.W. Aherne, *J. Pharm. Biomed. Anal.* **12**, 353–360 (1994).
[16] D.B. Predmore, G.D. Christian and T.A. Loomis, *J. Forensic Sci.* **23**, 481–489 (1978).
[17] C. Lora-Tamayo, T. Tena and G. Tena, *J. Chromatogr.* **422**, 267–273 (1987).
[18] R. Wasels, F. Belleville, P. Paysant and P. Nabet, *J. Chromatogr.* **489**, 411–418 (1989).
[19] E.J. Cone, W.D. Darwin and W.F. Buchwald, *J. Chromatogr.* **275**, 307–318 (1983).

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