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A BIOASSAY FOR INHIBITION OF SEROTONIN RELEASE FROM BOVINE PLATELETS

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ABSTRACT.—A bioassay was developed to study agents capable of inhibiting the release of serotonin from bovine blood platelets. It is a simple, inexpensive, and reproducible high-throughput bioassay suitable for quality control of feverfew, *Tanacetum parthenium*, a crude drug with proven migraine prophylactic activity that is being considered for governmental registration and regulation. The bioassay, which requires no experimental animals or human subjects, was used to assess the in vitro activity of *T. parthenium* samples grown from seed obtained from 10 different regions of Europe. The activity was found to vary significantly within and between samples, with no geographical correlation. Serotonin release inhibition was shown to be significantly correlated with the content of the germacranolide sesquiterpene lactone, parthenolide, although other sesquiterpene lactones from this plant and other members of the Asteraceae were also shown to be active. The activities of six other species of *Tanacetum*, as well as of *Artemisia absinthium* (wormwood) and *Zingiber officinale* (ginger), and two commercial drugs for migraine prophylaxis, verapamil hydrochloride and propranolol hydrochloride, were also assessed. The relevance of the bovine platelet serotonin release inhibition bioassay to antimigraine research is discussed.

The leaf of feverfew, *Tanacetum parthenium* (L.) Schultz-Bip. (Asteraceae), is a popular British traditional herbal remedy for the prophylaxis of migraine. Berry (1) reviewed its traditional European uses and described how in the late 1970's feverfew gained renewed popularity among sufferers of migraine and arthritis, due to published reports of its effectiveness in treating patients who did not respond well to orthodox therapies. Several reviews of the botany, phytochemistry, and pharmacology of feverfew have been published recently (2–5).

Two double-blind placebo-controlled clinical trials (6,7), the second of which had a randomized, cross-over design, demonstrated the efficacy of feverfew in reducing the frequency and severity of attacks of migraine with and without aura. No serious side effects of feverfew ingestion were observed, and no changes were seen in hematological tests, urea, creatinine, electrolytes, blood sugar, urinalysis, or tests of liver function (7). Feverfew has also been examined for its potential as an anti-inflammatory and antithrombotic drug (8–11).

This clinical evidence demonstrating the efficacy of feverfew as a migraine prophylactic, coupled with the identification of parthenolide as the presumed active

principle of the feverfew leaf used in the clinical trials (12–14), suggested that registration could be sought for feverfew products to be sold making specific therapeutic claims. It has been proposed that botanical preparations could be certified through a scheme based on independent verification of botanical identity, a specified minimum acceptable level of the presumed active principle(s) as determined by an unambiguous chemical analysis, and an in vitro biological assay for further standardization of commercial lots (5, 15–19). Such certified products would be subject to existing standard quality control procedures with regard to pesticide residues, extraneous matter, microbiological contamination, and adulterating substances (17).

Analysis by hplc and ^{1}H nmr for parthenolide content of feverfew indicated substantial variability among authenticated samples of *T. parthenium* (20,87). A preliminary assay of commercial preparations of feverfew by in vitro human platelet inhibition showed a wide variation in activity. Levels of inhibition were always lower than expected from the declared content of feverfew (21). We describe here the application of a relatively simple in vitro biological assay for inhibition of serotonin release from bovine blood platelets to the analysis of crude extracts of *T. parthenium*, selected other plant species, pure sesquiterpene lactones isolated from these plants, and two commercial drugs used for migraine prophylaxis, and discuss its relevance to research on migraine prophylaxis.

RESULTS

The bovine platelet bioassay for inhibition of 5-HT release provides a throughput of at least four samples (compounds or extracts) tested at five concentrations with three replicates per person per day, including preparation and cleanup time, at a cost of approximately $4 per sample. Required equipment such as a thrombocounter and scintillation counter are readily available, and scale-up requires only additional personnel. No experimental animals or human subjects are required. It therefore meets criteria for a simple, inexpensive, high-volume assay suitable for quality control or screening research.

Because the bovine platelet bioassay employs blood obtained as a byproduct of the meat processing industry, it may not be possible to select the age, sex, or breed of cattle used. This might be expected to introduce a significant amount of variability into the bioassay. To assess the reproducibility of the bioassay, the IC_{50} in μM (and 95% confidence limits) of pure parthenolide was determined on five different occasions: 1.78 (1.14–2.73), 3.34 (2.41–4.51), 2.67 (1.96–3.57), 4.50 (3.26–6.04), and 2.88 (2.09–3.89). The resulting mean IC_{50} of 3.03 μM had narrow 95% confidence limits (1.80–4.27 μM), indicating the bioassay is highly reproducible.

Platelets were exposed to [^{14}C]-5-HT (specific activity 54.5 mCi/mmol) at 0.05 μCi/ml of citrated blood. The amount of radioactivity remaining in the supernatant in the absence of inhibitor (parthenolide) or activator (adenosine diphosphate, ADP) (untreated control) was 24.9% (21.1–28.7, $n = 42$) of the total platelet-rich plasma (PRP) radioactivity corrected for dilution, indicating that approximately 75% of the [^{14}C]-5-HT was taken up by the platelets. The amount of radioactivity present in the supernatant of the positive controls (parthenolide + ADP) was 20.3% (16.7–23.9) of the corrected total, which suggested parthenolide (100 μM) inhibits even some random release of 5-HT, but a Tukey pairwise comparison of means indicated no significant difference between untreated and positive controls at a rejection level of 0.05. Treatment of platelets with ADP (100 μM) in the absence of inhibitor (negative control) resulted in an average release of 29.8% (25.5–34.1) of that fraction of the total radioactivity present in the platelets (value corrected for dilution and untreated control).

A screening of 5-HT release inhibition by crude EtOH extracts of *T. parthenium* samples from 10 different seed sources (5 replicates of each) is presented in Table 1.

TABLE 1. Serotonin Release Inhibition by *Tanacetum parthenium* from Different Geographic Locations.

Lot no.	Source of seed (all cultivated at Agriculture Canada, Ottawa)	Mean IC_{50} (95% conf. lim.) (μg/ml, fresh wt)
CC1	Botanical Garden of Belgium, Brussels	1357 (2670–4444)[a]
CC3	University of Bayreuth, Germany	10540 (6024–15050)[bc]
CC5	Botanical Garden, Bremen, Germany	3879 (1207–6551)[ab]
CC9	Botanical Garden of Nancy, France	3431 (2343–4518)[a]
CC10	University of Nottingham, England[c]	2937 (2513–3362)[a]
CC13	Botanical Garden of Geneva, Switzerland	8845 (3238–14450)[abc]
CC14	Botanical Garden of Caen, France	18910 (12920–24910)[d]
CC19	Botanical Garden of Palermo, Sicily	12090 (7279–16910)[cd]
CC25	University of Genoa, Italy	2696 (0–6887)[a]
CC28	Newcastle-upon-Tyne, England	8801 (0–22840)[abc]

[a–d]Superscripts indicate groups of means not significantly different from one another as determined by Tukey pairwise comparisons of means, with a rejection level of 0.05.

[c]Nottingham sample is the standard for comparative evaluation of other extracts: less than 3-fold different (IC_{50}<8811 μg/ml) = strongly active; between 3- and 6-fold greater (8811<IC_{50}<17622 μg/ml) = moderately active; more than 6-fold greater (IC_{50}>17622 μg/ml) but still measurable = weakly active.

There are significant differences in the biological activity of specimens of the same species obtained from different sources. One-way analysis of variance followed by Tukey's pairwise comparisons of means indicated four groups (Table 1) in which the means are not significantly different from one another at a rejection level of 0.05. The highest level of activity was seen in the samples obtained from Genoa (seed lot CC25), whose hplc chromatogram showed a number of major peaks in addition to that of parthenolide. The least active samples came from Caen (CC14, several replicates of which were almost completely inactive at the highest concentration tested) and from Palermo (CC19). The hplc chromatograms of CC1, CC5, CC10, and CC28 indicated that parthenolide was the major sesquiterpene lactone present. The chromatograms of CC3, CC9, CC13, CC14, and CC19 indicated lower levels of sesquiterpene lactones with other peaks approaching a similar integration to that of parthenolide, which was nevertheless readily detectable.

Due to the extensive phytochemical, pharmacological, and clinical studies conducted on feverfew samples from the University of Nottingham (7–14), this sample was selected as a standard for comparative evaluation of other crude extracts. Based approximately upon the statistical significance of the differences of their means, extracts with an IC_{50} less than threefold different (IC_{50}<8811 μg/ml) were considered strongly active, those whose IC_{50} lay between threefold and sixfold greater (8811<IC_{50}<17622 μg/ml) were considered moderately active, and extracts with IC_{50} values more than sixfold greater (IC_{50}>17622 μg/ml) but still showing measurable activity were considered weakly active.

A number of other species of *Tanacetum* were also grown from seed and assayed for bovine platelet 5-HT release inhibition (Table 2). Two other plants were also bioassayed: (1) wormwood (absinthe) leaves (*Artemisia absinthium* L.) another genus of the Asteraceae containing sesquiterpene lactones (but not α,β-unsaturated) (22), used as a traditional remedy for migraine (23), and (2) ginger rhizome (*Zingiber officinale* Rosc., Zingiberaceae), a plant totally unrelated to the Asteraceae, containing sesquiterpenes (but non-lactone), also used traditionally for migraine prophylaxis and therapy (24–26). The ginger constituents, 6-, 8-, and 10-gingerol, phenolic constituents with a diketone function in the sidechain, have been shown to have 5-HT_3 receptor antagonist

TABLE 2. Serotonin Release Inhibition by Other Species of Plants.

Lot no.	Scientific name and source of seed or material	Growth stage	IC_{50} (95% conf. lim.)[a] (μg/ml, fresh wt)
CC2	*Tanacetum sericeum* (Adams) Schultz-Bip. University of Bayreuth, Germany	full anthesis	>21000 (inactive)
CC4	*Tanacetum corymbosum* (L.) Schultz-Bip. ssp. *clusii* (Fisher ex Reichenb.) Heywood University of Bayreuth, Germany	full anthesis	37686 (7640–∞)
CC4	*T. corymbosum* (L.) Schultz-Bip. ssp. *clusii* (Fischer ex Reichenb.) Heywood University of Bayreuth, Germany	preanthesis	>19000 (inactive)
CC7	*Tanacetum vulgare* L. Wissenschaften DDR, Germany	full anthesis	9797 (6207–∞)
CC8	*Tanacetum poteriifolium* (Ledeb.) Grierson Wissenschaften DDR, Germany	full anthesis	>20000 (inactive)
CC8	*T. poteriifolium* (Ledeb.) Grierson Wissenschaften DDR, Germany	preanthesis	>17000 (inactive)
CC22	*Tanacetum siculum* P.G. Strobl Botanical Garden of Palermo, Sicily	full anthesis	13877 (9165–24038)
CC23	*T. corymbosum* (L.) Schultz-Bip. Humboldt University, Berlin, Germany	full anthesis	>22000 (inactive)
CC27	*T. corymbosum* (L.) Schultz-Bip. J.-M. Aubert F., Champex-Lac, Valais, Switzerland	preanthesis	>27000 (inactive)
CC32	*Tanacetum macrophyllum* (W. & K.) C.H. Schultz Oldenburg University, Germany	preanthesis	>26000 (inactive)
CC34	*T. vulgare* L. Oldenburg University, Germany	preanthesis	>26000 (inactive)
RM1	*Artemisia absinthium* L. Agriculture Canada, Ottawa	full anthesis	>15000 (inactive)
RM2	*Zingiber officinale* Rosc. Local market, Ottawa	commercial rhizome	>25000 (inactive)

[a]Standard for comparative evaluation of extracts: IC_{50}<8811 μg/ml = strongly active; 8811<IC_{50}<17622 μg/ml = moderately active; IC_{50}>17622 μg/ml but still measurable = weakly active.

activity (27), which would explain the reported antiemetic activity and possible efficacy in the symptomatic treatment of migraine (28).

From Table 2 it can be seen that one sample of *Tanacetum vulgare* L. from Germany (CC7) and a sample of *Tanacetum siculum* P.G. Strobl from Sicily (CC22) showed moderate levels of 5-HT release inhibition, while *Tanacetum corymbosum* (L.) Schultz-Bip. ssp. *clusii* (Fischer ex Reichenb.) Heywood from Germany (CC4) was weakly active. Interestingly, another sample of *T. vulgare* from Germany (CC34) was inactive at the highest concentration tested (26 mg/ml), emphasizing the variability of the phytochemistry of this genus. Both *A. absinthium* and *Z. officinale* were inactive at the maximum concentrations tested (15 mg/ml and 25 mg/ml, respectively), although the well-known toxicity of wormwood, attributed to its thujone content (29), should discourage its use in any case. All of these plant extracts were determined by hplc analysis to have no detectable parthenolide content, so the activity of the above *Tanacetum* species must be due to other constituents, possibly other sesquiterpene lactones.

Some of the averages in Table 1 have broad 95% confidence intervals, indicating significant differences between replicates from the same seed lot, despite the fact that they were of identical age and were grown under identical conditions. Because the reproducibility of the bioassay was clearly established, variability in parthenolide content of the samples was checked by hplc and compared with the content of sesquiterpene lactones calculated as parthenolide equivalents from the IC_{50} of the crude extract and the

IC_{50} of pure parthenolide. The results of analysis of 25 samples are presented in Table 3, and the correlation of the bioassay versus hplc assay is presented in Figure 1. The correlation was highly significant ($r = 0.95$, $p < 0.001$) and accounted for 90% of the observed variance ($r^2 = 0.90$).

The bioassay generally detected less "sesquiterpene lactones calculated as parthenolide equivalents" than hplc analysis did, suggesting that the bioassay is less sensitive than hplc analysis. Overall, however, the mean results of the two methods are not significantly different according to a Tukey comparison of means. Comparison of the means of the bioassay/hplc ratios for each of the lots of *T. parthenium* in Table 3 revealed two extremes, CC13 and CC14, that were significantly different from each other but not from the rest of the samples (Tukey test, rejection level 0.05). Their hplc chromatograms for sesquiterpene lactone detection were qualitatively and quantitatively very similar. The low extreme of CC14, with a mean ratio of 0.36, may represent a plant with an interfering constituent (e.g., a secretion stimulant), which has been reported previously (14). The other extreme, CC13, with a mean ratio of 0.96 and three samples with a ratio of >1, suggested the possibility that other constituents of the plant were contributing to the 5-HT release inhibition.

A review of the phytochemical literature on *T. parthenium* revealed that this species is known to contain at least 30 sesquiterpenes, 11 monoterpenes, 6 spirofurans, and 1 flavonoid (13, 30–39). Of these, in addition to parthenolide, four sesquiterpene lactones, 3-β-hydroxyparthenolide, *seco*-tanapartholide A, canin, and artecanin, have been reported to inhibit 5-HT release from human platelets (13). Using the bovine

TABLE 3. Parthenolide Content of *Tanacetum parthenium* Samples as Determined by Hplc and Bovine Blood Platelet Bioassay.

Voucher no.	Source of seed	Parthenolide (%) (fresh wt basis)	
		hplc	bioassay
CC1C	Botanical Garden of Belgium, Brussels	0.0264	0.0260
CC3A	University of Bayreuth, Germany	0.0100	0.0047
CC3B	University of Bayreuth, Germany	0.0115	0.0073
CC3C	University of Bayreuth, Germany	0.0139	0.0079
CC3D	University of Bayreuth, Germany	0.0150	0.0126
CC3E	University of Bayreuth, Germany	0.0095	0.0069
CC5B	Botanical Garden, Bremen, Germany	0.0361	0.0253
CC9B	Botanical Garden of Nancy, France	0.0135	0.0153
CC10D	University of Nottingham, England	0.0302	0.0262
CC13A	Botanical Garden of Geneva, Switzerland	0.0083	0.0101
CC13B	Botanical Garden of Geneva, Switzerland	0.0122	0.0131
CC13C	Botanical Garden of Geneva, Switzerland	0.0149	0.0195
CC13D	Botanical Garden of Geneva, Switzerland	0.0092	0.0054
CC13E	Botanical Garden of Geneva, Switzerland	0.0091	0.0057
CC14A	Botanical Garden of Caen, France	0.0113	0.0040
CC14B	Botanical Garden of Caen, France	0.0150	0.0056
CC14C	Botanical Garden of Caen, France	0.0102	0.0028
CC14D	Botanical Garden of Caen, France	0.0121	0.0044
CC14E	Botanical Garden of Caen, France	0.0091	0.0040
CC19B	Botanical Garden of Palermo, Sicily	0.0123	0.0085
CC19C	Botanical Garden of Palermo, Sicily	0.0090	0.0053
CC19D	Botanical Garden of Palermo, Sicily	0.0091	0.0074
CC19E	Botanical Garden of Palermo, Sicily	0.0132	0.0050
CC25C	University of Genoa, Italy	0.0577	0.0492
CC28B	Newcastle-upon-Tyne, England	0.0339	0.0308

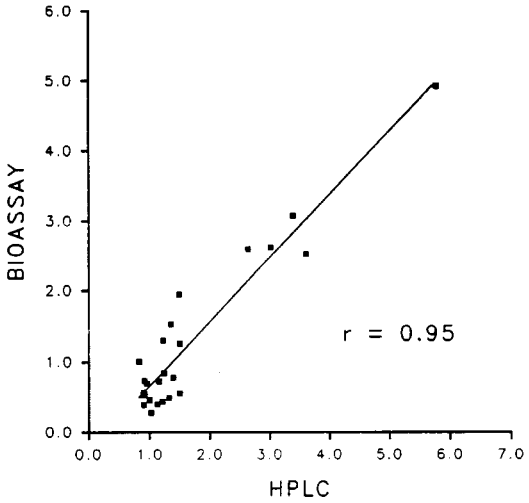

FIGURE 1. Correlation between the parthenolide content of *Tanacetum parthenium* samples determined by hplc versus content of sesquiterpene lactones calculated as parthenolide equivalents from the bioassay IC_{50} of the crude leaf EtOH extract and the IC_{50} of pure parthenolide. Units are expressed as percent fresh weight × 100.

platelet bioassay, activities were assessed for ten well-known sesquiterpene lactones from *T. parthenium* and other members of the Asteraceae, representing each of the four main skeletal types (Table 4). The activities of these sesquiterpene lactones contrast very favorably with the 5-HT release inhibition of two commercial drugs used therapeutically or experimentally in migraine prophylaxis: verapamil hydrochloride and propranolol hydrochloride (Table 4). Structure-activity relationship analysis for a series of more than 50 pure sesquiterpene lactones is currently in progress.

The results given in Table 4 confirm that while parthenolide was identified by antisecretory bioassay-guided fractionation as the major active sesquiterpene lactone of the feverfew used in the British clinical trials, other sesquiterpenes with an α,β-unsaturated lactone function contribute to feverfew activity (13,40). Saturation of the exocy-

TABLE 4. Serotonin Release Inhibition by Pure Compounds.

Compound name	Class	IC_{50} μM (95% limits)
Verapamil hydrochloride	Calcium channel blocker	577.5 (269.4–1952.7)
Propranolol hydrochloride	β-Adrenergic blocker	>939.8
Parthenolide	Germacranolide	3.03 (1.80–4.27)
11β,13-Dihydroparthenolide	Germacranolide	>399.5
1,10-Epoxycostunolide	Germacranolide	121.3 (85.42–179.3)
Cnicin	Germacranolide	3.52 (2.49–4.87)
Reynosin	Eudesmanolide	271.1 (91.12–495.9)
Santamarin	Eudesmanolide	84.93 (52.27–151.7)
α-Santonin	Eudesmanolide	>568.4
Isoalantolactone	Eudesmanolide	>516.5
Parthenin	Pseudoguaianolide	129.3 (81.41–225.8)
Artecanin	Guaianolide	>134.7

clic methylene in 11β,13-dihydroparthenolide (Table 4) resulted in a complete loss of activity up to the maximum concentration tested (400 μM). Specimens of *T. parthenium* from Yugoslavia, Mexico, and Guatemala have been found devoid of parthenolide but rich in other sesquiterpene α-methylene butyrolactones, including santamarin and reynosin (5). A sample of dried *T. parthenium* leaves from Guatemala, which was determined by hplc analysis to have no detectable level of parthenolide, had a bovine platelet bioassay IC_{50} (dry-wt based) of 1031 μg/ml (826–1250). While direct comparison of dry-wt versus fresh-wt based values is difficult due to varying amounts of moisture in growing plants, this sample clearly has a level of activity on a par with some of the European *T. parthenium* samples, which may be due to sesquiterpene lactones such as santamarin and reynosin. Reynosin has been reported from *T. parthenium* growing in Germany (30), and although we have not yet conclusively identified other active principles in sample CC25 from Italy, the retention times and absorption spectra of peaks in its chromatogram are similar to those of reynosin and santamarin.

DISCUSSION

The bovine platelet bioassay provides a convenient means for determining the ability of both pure compounds and crude plant extracts to inhibit the release of 5-HT from platelets in vitro. Serotonin undoubtedly plays a significant role in the pathogenesis of migraine headaches. Platelets from migraine sufferers have significantly higher spontaneous aggregation and adhesion, and greater sensitivity to 5-HT-releasing agents such as adrenaline, than those of non-sufferers. However, the precise roles of platelets and 5-HT, as well as distinctions between causes and effects, are quite controversial (41–46). Migraine pathogenesis has been reviewed recently by Appenzeller (47) and Diamond (48).

A mechanism of action of feverfew in migraine prophylaxis has been suggested by several in vitro studies. Collier *et al.* (49) reported that feverfew extracts inhibited prostaglandin biosynthesis in vitro, but not through acetylsalicyclic acid's mechanism of cyclooxygenase inhibition. Makheja and Bailey (50,51) found that in vitro platelet aggregation induced by ADP, collagen, or thrombin was prevented by feverfew extracts, and they concluded that feverfew's inhibition of prostaglandin biosynthesis was through inhibition of phospholipase A_2. Hayes and Foreman (52) showed that feverfew extracts also inhibited secretion of histamine from mast cells. Heptinstall *et al.* (12) reported that secretory activity in polymorphonuclear leucocytes was also inhibited, and that the mechanism was probably not through phospholipase inactivation. Sesquiterpene lactones containing an α-methylene butyrolactone, including parthenolide, and feverfew extracts caused significant depletion of intracellular reduced glutathione, resulting in changes in arachidonic acid metabolism, and alkylation of protein sulfhydryl groups, through Michael-type nucleophilic addition (40). Parthenolide and feverfew extracts inhibited the secretion of 5-HT but not platelet aggregation induced by phorbol-12-myristate-13-acetate, suggesting a possible interaction of parthenolide with protein kinase C (13,14).

Relating the results of in vitro experiments to effects in vivo is always problematic due to the complexity of interconnected metabolic systems in living organisms. Caution must be exercised in such extrapolations. In a limited study of only ten patients who were chronic feverfew users, platelet aggregation in six patients who had recently taken feverfew was greatly attenuated in response to 5-HT and the synthetic thromboxane A_2-mimetic, U46619, but not in response to ADP or thrombin, when compared with four patients who had stopped taking feverfew at least six months previously (53). On this basis, Biggs *et al.* (53) questioned the applicability to the clinical situa-

tion of Makheja and Bailey's (50) results from the addition of aqueous extracts of feverfew to platelets in vitro. Because the study of Biggs *et al.* (53) was not conducted on a statistically significant number of patients, no data were presented on the dosage, time course, or source of feverfew, which is known to be highly variable in its content of the presumed active principles (20,21), and because the pharmacokinetics of the active principles of feverfew are unknown, it is difficult to assess the true significance of their findings. Nevertheless, feverfew consumption has been clinically proven to reduce the incidence and severity of migraine (6,7) and was shown to affect patients' platelet functions (53). The decrease in platelet 5-HT levels is the most consistent and relatively specific to migraine of all the biological alterations observed in attacks of migraine (46). While platelet release of 5-HT may be more a reflection of a systematic abnormality of 5-HT metabolism than of direct consequence to migraine, the platelet has been considered as a model for a pathophysiologically more important but inaccessible system, such as the serotonergic neuronal projections within the CNS and elsewhere (46). Thus, until the pharmacology and toxicology of feverfew constituents are better known, in vitro studies on well-established models such as platelet release inhibition are the only practical and ethical methods for scientific investigation.

Although human platelets are obviously the most relevant to investigations of antimigraine pharmacology, obtaining sufficient quantities of fresh blood on a regular basis presented logistic problems. University ethical and health safety guidelines require blood donors to be anonymous and screened for AIDS and hepatitis. For labeling the platelets with [^{14}C]-5-HT by an active-transport uptake process, the blood has to be fresh. Screening large numbers of plant extracts and pure compounds required up to 240 ml of fresh blood per day for an extended period. Thus existing blood banks could not be used, and establishing a large enough group of suitable donors to avoid too frequent requests for blood was deemed impractical.

Use of animal blood was therefore considered, but due to the volumes needed and concomitant ethical and economic considerations, the use of common small laboratory mammals was also not practical. Large domestic mammals are the most practical source of quantities of fresh blood, which can be obtained as a byproduct of the meat-processing industry and would normally be discarded. Selection of an appropriate animal for platelet release studies was problematic because human platelet behavior is significantly different from all other species, including other primates (54,55). After reviewing the literature on comparative hematology, the cow was chosen as the source of blood for these experiments because of its large size, ready availability, and hematological similarities with humans, including insensitivity of platelet aggregation to extracellular Ca^{2+} concentration, irreversible aggregation response to ADP, similar platelet dense granule constituents and their ratios, and similar platelet membrane major proteins and glycoproteins (54–56). Use of bovine blood from the slaughterhouse has also helped reduce the use of experimental animals, of which almost two million were used in research, teaching, and testing in Canada alone in 1989 (57).

Obviously, with the cow or any other animal, caution will be required when extrapolating to effects on human platelets, but, with appropriate control studies, the use of bovine platelets has proven to be an effective substitute in the in vitro model of inhibition of 5-HT release. In the 5-HT release inhibition bioassay using bovine platelets, the IC_{50} for parthenolide was 3.03 μM (1.80–4.27), and for the migraine prophylactic drug verapamil hydrochloride it was 577.5 μM (269.4–1952.7), while substituting human platelets under identical experimental conditions gave IC_{50} values for parthenolide of 5.64 μM (2.78–9.71) and for verapamil hydrochloride 770.2 μM (350.2–2746.1). Thus there was no significant difference between human and bovine platelet reactivity at a rejection level of 0.05.

For regulatory purposes, the bovine platelet bioassay will be useful only if employed in conjunction with a phytochemical assay and botanical authentication, since an extensive review of the literature (58) indicates the presence of parthenolide in 25 species (10 genera) of the Asteraceae and 9 species (3 genera) of the Magnoliaceae. Five non-asteraceous plant species have previously been reported as 5-HT secretion inhibitors, and 22 natural products have been reported to inhibit the release of 5-HT: 12 flavonoids, 6 sesquiterpenes, 2 quinoids, 1 triterpene, and 1 coumarin (58). The wide variety of plant species and natural products reported to inhibit platelet 5-HT release indicates that a variety of mechanisms must be involved. The pathways of platelet activation operate synergistically, so an inhibitor of one pathway may appear to inhibit several others (59).

Drugs currently available for the prophylaxis of migraine have been the subject of several recent reviews (46, 48, 60–62). They include β-adrenergic blockers (e.g., propranolol), α-adrenergic agonists (e.g., clonidine), calcium-channel blockers (e.g., verapamil), monoamine oxidase inhibitors (e.g., phenelzine), non-steroidal anti-inflammatory drugs (e.g., naproxen sodium, aspirin), cyclic AMP phosphodiesterase inhibitors (e.g., caffeine), 5-HT uptake inhibitors (e.g., amitriptyline), 5-HT_{1A} receptor agonists (e.g., dihydroergotamine), 5-HT_{1D} agonists (e.g., sumatriptan), and 5-HT_2 or 5-HT_{1C} antagonists (e.g., pizotifen). The only common feature of all drugs effective in both prophylactic and acute migraine therapy is that they stabilize serotonergic neurotransmission (46). There is a delayed response to migraine prophylaxis with feverfew (5,6). This has also been observed with propranolol, verapamil, amitriptyline, methysergide, and cyproheptadine, all of which exhibit 5-HT_2 antagonism, and for which the delayed response was attributed to down-regulation of cerebral 5-HT receptors (46).

Inhibition of platelet aggregation and 5-HT release by verapamil and propranolol has been reported (48). The presynaptic release of 5-HT and other neurotransmitters is calcium-dependent (46). In the bovine platelet bioassay, verapamil hydrochloride was 100-fold less active than parthenolide (Table 4). Propranolol hydrochloride, which blocks catecholamine-induced platelet aggregation as well as being a 5-HT_2 antagonist (48), was inactive against ADP-induced bovine platelet aggregation at concentrations up to 278 μg/ml (939.8 μM, Table 4). The established migraine prophylactic patient dosage for verapamil is 360 mg per day, and for propranolol it is 40 mg four times per day (48).

Bioassays for inhibition of 5-HT release from platelets might be more selective than simple platelet aggregation inhibition assays such as the modified smear method of Yun-Choi *et al.* (63), but clearly more biochemically-oriented bioassays, such as specific enzyme activity modulation and receptor binding assays, will be required to understand the mechanism of action of natural products with potential antimigraine or antithrombotic applications.

The pharmaceutical use of feverfew and its sesquiterpene lactones, especially for migraine, arthritis, and bronchial complaints, has already been patented (64). However, because more than 60 plant species are reported in the literature as traditional medicines specifically for migraine headaches (23–26, 65–80), there seems to be a reasonable probability of discovering new natural products with therapeutic and economic potential.

We are now assessing a number of pure sesquiterpene lactones for potential antimigraine activity, using the bovine platelet bioassay as a prescreen to select compounds of particular interest. Through biochemical mechanistic studies and quantitative structure-activity relationship analysis it may be possible to make therapeutically

useful distinctions between the potential antimigraine activity of sesquiterpene lactones and their well-known cytotoxic (81,82) and allergenic properties (32,83,84).

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—Blood samples were centrifuged in an IEC Clinical Centrifuge (International Equipment, Needham Heights, MA). Platelet counts were made with a Coulter Thrombocounter-C (Coulter Electronics, Hialeah, FL), for which saline (Isoton II) and cuvettes (20 ml) were obtained from Coulter Electronics of Canada Ltd., Burlington, Ontario. Thrombocounter calibration was checked with Streck Platelet Chex Tri-Pack (Streck Laboratories, Omaha, NE). Centrifugation of assay tubes was with a Fisher Microcentrifuge Model 59 (Fisher Scientific, Nepean, Ontario). Scintillation counting used scintillation cocktail Scintran EX (BDH, Inc., Toronto) with a Packard 2000CA Tri-Carb Liquid Scintillation Analyzer (Packard Instrument, Downers Grove, IL). Analysis by hplc was performed with a system consisting of an SP8700 pump (Spectra Physics, Mississauga, Ontario), a Valco CW6 injector (Valco Instruments Co., Houston, TX), and a Pharmacia-LKB 214O RSD diode-array detector (Pharmacia-LKB, Baie d'Urfe, Quebec), using a Brownlee Spheri-10 RP-18 column (250 mm × 4.6 mm × 10 μm, Applied Biosystems, Santa Clara, CA). Quantitative data evaluation was conducted on an IBM-AT computer using Pharmacia-LKB Wavescan-EG and Nelson Analytical 2600 Chromatography Software. All chemicals were analytical grade and all solvents were chromatography grade (BDH). Adenosine-5′-diphosphate (ADP) Sodium salt, Grade IX, acetylsalicyclic acid (ASA), verapamil hydrochloride, and propranolol hydrochloride were obtained from Sigma Chemical, St. Louis, MO. 5-[2-^{14}C]-Hydroxytryptamine binoxalate ([^{14}C]-5-HT, 54.5 mCi/mmol, 0.1 mCi/ml), was obtained from New England Nuclear/Dupont Canada, Mississauga, Ontario.

PLANT MATERIAL.—Seed for various species of *Tanacetum* was obtained from botanical gardens in different locations throughout Europe (see Table 1), and cultivated under identical conditions at the Central Experimental Farm, Agriculture Canada, Ottawa, Ontario. Leaf samples were collected during early to full anthesis and immediately placed in EtOH (95%). Separate samples were collected from each of five plants for each seed lot. Voucher specimens were identified by C. Crompton and deposited in the herbarium of the Centre for Land and Biological Resources Research, Research Branch, Agriculture Canada, Ottawa.

PURE ISOLATES.—Parthenolide was obtained by extraction and isolation from an authenticated commercial bulk sample of *T. parthenium* leaf powder, using the methods of Bohlmann and Zdero (30). It was identified spectroscopically and by comparison with an authentic sample obtained from the collection of the Chemistry Department, Louisiana State University. The other pure sesquiterpene lactones were obtained from the same collection and also that of the Biology Department, University of Ottawa. Purity of the isolates was verified by hplc prior to bioassay.

PREPARATION OF PLANT EXTRACTS AND PURE COMPOUNDS.—Fresh leaf material (30.6 g average) was macerated with EtOH (95%, 200 ml) for 24 h, filtered under vacuum, concentrated to a syrup under reduced pressure at 40°, and then redissolved in EtOH (95%, 10 ml). This solution was filtered first through cotton wool and then through a membrane filter (0.8 μm). Samples were divided into two portions, one for bioassay and one for hplc analysis. This method of extraction was determined by hplc analysis to give concentrations of parthenolide comparable to those obtained by exhaustive Soxhlet extraction with petroleum ether. Pure compounds (2.0 mg) were dissolved in EtOH (95%, 100 μl) to make a 20 mg/ml stock solution. Serial dilutions were prepared in EtOH (95%). Aliquots of extract or compound (30 μl) were added to PBS (970 μl) and vortexed to make assay solutions. Using 100 μl in a total assay volume of 600 μl gave a final concentration of 0.5% EtOH.

ANALYSIS BY HPLC.—EtOH extracts were analyzed by direct injections (20 μl) onto a reversed-phase hplc system, using an RP-18 column (250 mm × 4.6 mm × 10 μm) with diode-array uv detection at 210 nm. Elution was isocratic with a mobile phase of MeCN-H_2O (45:55) at a flow rate of 2.0 ml/min (900 psi). Quantitative results were obtained using parthenolide (Rt 5.9 min) as an external standard in MeCN solutions ranging in concentration from 50 to 2000 μg/ml (r^2>0.999) (20). Two replicates of each assay were performed and the results averaged.

DESCRIPTION OF THE BIOASSAY.—Beef cattle at the slaughterhouse were restrained and stunned, then bled from the throat. Arterial blood was added to a calibrated plastic bottle (120 ml) containing trisodium citrate (12 ml, 0.1 M) as an anticoagulant and [^{14}C]-5-HT (6.0 μCi), and transported in a vacuum flask to prevent excessive cooling. Platelets rapidly take up [^{14}C]-5-HT by a high affinity active transport process (85,86). The total elapsed time between the collection of the blood and the start of the assay was usually approximately 90 min.

Serotonin release inhibition was determined following the methodology of Heptinstall *et al.* (12). Platelet-rich plasma (PRP) was prepared by centrifugation (180*g*, 15 min) and diluted to 300,000 platelets/μl with autologous platelet-poor plasma, prepared by further centrifugation of the blood (630*g*, 10 min). Tubes containing 3% EtOH in phosphate-buffered saline (PBS) (100 μl) served as untreated controls (no sample, no aggregating agent) and negative controls (no sample, with aggregating agent), and tubes containing parthenolide (final concentration 100 μM) served as a positive control. Results were averaged over three treatment replicates and 2 control replicates.

Aliquots of PRP (450 μl) were preincubated with extracts, compounds, or controls (100 μl) for 3 min at 37° under an atmosphere of 5% CO_2 in air, with magnetic stirring (1000 rpm). Then PBS (50 μl) was added to the untreated controls, and the aggregating agent ADP (50 μl, final concentration 100 μM) was added to the rest of the tubes. After 6 min incubation as above, the aggregation and release reaction was stopped by adding ice-cold acetylsalicyclic acid (50 μl, final concentration 1.0 μM), capping and shaking each tube, and placing them on ice. The samples were then centrifuged (3000 *g*, 10 min), and aliquots (50 μl) of the supernatant were subjected to scintillation counting. An aliquot of EtOH (3% in PBS) was used as a blank and aliquots of PRP (2 replicates) were used to determine the total radioactivity.

Inhibition of [^{14}C]-5-HT release in each sample was calculated from the percentage of the total radioactivity released into the supernatant in the negative control (i.e., maximal release in the presence of ADP and absence of inhibitor), with corrections for dilution and the untreated control count (indicating the amount of [^{14}C]-5-HT not taken up by the platelets, or released randomly or by handling and stirring). The concentration of sample resulting in a 50% inhibition of [^{14}C]-5-HT release (IC_{50}) and the 95% confidence limits were calculated by probit analysis of percent inhibition versus log concentration. For pure compounds the IC_{50} was converted from μg/ml to μM units.

Experiments were not continued once the total elapsed time since collection of the blood exceeded 3 h, because platelets lose their reactivity with time. Controls were included with every second set of samples in order to monitor platelet reactivity. In instances where an observed dpm count was lower than the untreated control or higher than the negative control, these values were substituted to normalize the calculations to 0–100%. Each sample was assayed twice, first at logarithmic dilutions for an initial estimate of activity, then at appropriate geometric dilutions for a more precise determination of the IC_{50}.

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